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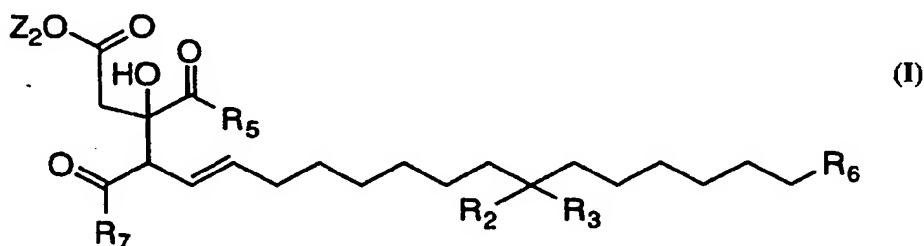
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(54) Title: BIOLOGICALLY ACTIVE COMPOUNDS ISOLATED FROM AEROBIC FERMENTATION OF *TRICHODERMA VIRIDE*



(57) Abstract

This invention relates to compounds of structural formula (I) isolated from an aerobic fermentation of *Trichoderma viride* MF5628, ATCC 74084, which are squalene synthase inhibitors and thus useful as cholesterol lowering agents. These compounds are also potent antifungal agents. Additionally, they inhibit farnesyl protein transferase and farnesylation of the oncogene protein Ras and are thus useful in treating cancer. This invention also relates to a process for obtaining compounds of structural formula (I).

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- 1 -

TITLE OF THE INVENTION
BIOLOGICALLY ACTIVE COMPOUNDS ISOLATED FROM
AEROBIC FERMENTATION OF TRICHODERMA VIRIDE

BACKGROUND OF THE INVENTION

5 Hypercholesterolemia is known to be one of the prime risk factors for ischemic cardiovascular disease, such as arteriosclerosis. Bile acid sequestrants have been used to treat this condition; they seem to be moderately effective but they must be consumed in large quantities, i.e. several grams at a time and they are not very palatable.

10 MEVACOR® (lovastatin) and ZOCOR® (simvastatin), now commercially available, are members of a group of very active antihypercholesterolemic agents that function by limiting cholesterol biosynthesis by inhibiting the enzyme HMG-CoA reductase.

15 Squalene synthase (also called squalene synthetase) is the enzyme involved in the first committed step of the de novo cholesterol biosynthetic pathway. This enzyme catalyzes the reductive dimerization of two molecules of farnesyl pyrophosphate to form squalene. The inhibition of this committed step to cholesterol should leave unhindered biosynthetic pathways to ubiquinone, dolichol and isopentenyl t-RNA.

20 Previous efforts at inhibiting squalene synthase have employed pyrophosphate or pyrophosphate analog containing compounds such as those described in P. Ortiz de Montellano *et al.*, J. Med. Chem. 20, 243 (1977), E.J. Corey and R. Volante, J. Am. Chem. Soc., 98, 1291 (1976), and U.S. Patent 5,025,003 to S. Biller. U.S. Patent 4,871,721 to S. Biller describes isoprenoid(phosphinylmethyl) phosphonates as inhibitors of squalene synthase.

25 U.S. Patents 5,096,923; 5,026,554; and 5,102,907 and U.S. Patent Applications Serial Numbers 496,734 filed March 21, 1990, and 698,766 filed May 10, 1991 disclose non-phosphorus-containing substituted 2,8-dioxabicyclo[3.2.1]octane derivatives useful as squalene synthase inhibitors. Furthermore, J. Antibiotics 45: 639-658 (1992) describe a squalene synthase inhibitor called squalastatin.

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- 2 -

Recently it has been shown that certain natural product nonphosphorous containing inhibitors of squalene synthase and their esters are useful in inhibiting fungal growth. This utility is described in U.S. Pat No. 5,026,554.

5 The present invention is directed to the use of compounds of structural formula (I) which are squalene synthase inhibitors for the inhibition of fungal growth.

10 The present invention is also directed to the use of compounds of structural formula (I) which are inhibitors of farnesyl protein transferase for inhibition of farnesylation of the oncogene protein Ras and the treatment of cancer.

15 The Ras gene is found activated in many human cancers, including colorectal carcinoma, exocrine pancreatic carcinoma, and myeloid leukemias. Biological and biochemical studies of Ras action indicate that Ras functions like a G-regulatory protein, since Ras must be localized in the plasma membrane and must bind with GTP in order to transform cells (Gibbs, J. et al., Microbiol. Rev. 53:171-286 (1989)). Forms of Ras in cancer cells have mutations that distinguish the protein from Ras in normal cells.

20 At least 3 post-translational modifications are involved with Ras membrane localization, and all 3 modifications occur at the C-terminus of Ras. The Ras C-terminus contains a sequence motif termed a "CAAX" or "Cys-Aaa1-Aaa2-Xaa" box (Aaa is an aliphatic amino acid, the Xaa is any amino acid) (Willumsen et al., Nature 310:583-586 (1984)). Other proteins having this motif include the Ras-related GTP-binding proteins such as Rho, fungal mating factors, the nuclear lamins, and the gamma subunit of transducin.

25 Farnesylation of Ras by the isoprenoid farnesyl pyrophosphate (FPP) occurs in vivo on Cys to form a thioether linkage (Hancock et al., Cell 57:1167 (1989); Casey et al., Proc. Natl. Acad. Sci. USA 86:8323 (1989)). In addition, Ha-Ras and N-Ras are palmitoylated via formation of a thioester on a Cys residue near a C-terminal Cys farnesyl acceptor (Gutierrez et al., EMBO J. 8:1093-1098 (1989); Hancock et al., Cell 57:1167-1177 (1989)). Ki-Ras lacks the palmitate

- 3 -

acceptor Cys. The last 3 amino acids at the Ras C-terminal end are removed proteolytically, and methyl esterification occurs at the new C-terminus (Hancock *et al.*, *ibid*). Fungal mating factor and mammalian nuclear lamins undergo identical modification steps (Anderegg *et al.*, *J. Biol. Chem.* 263:18236 (1988); Farnsworth *et al.*, *J. Biol. Chem.* 264:20422 (1989)).

Inhibition of Ras farnesylation *in vivo* has been demonstrated with lovastatin (Merck & Co., Rahway, NJ) and compactin (Hancock *et al.*, *ibid*; Casey *et al.*, *ibid*; Schafer *et al.*, *Science* 245:379 (1989)). These drugs inhibit HMG-CoA reductase, the rate limiting enzyme for the production of polyisoprenoids and the farnesyl pyrophosphate precursor. It has been shown that a farnesyl-protein transferase using farnesyl pyrophosphate as a precursor is responsible for Ras farnesylation. (Reiss *et al.*, *Cell*, 62:81-88 (1990); Schaber *et al.*, *J. Biol. Chem.*, 265:14701-14704 (1990); Schafer *et al.*, *Science*, 249:1133-1139 (1990); Manne *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:7541-7545 (1990)).

Inhibition of farnesyl-protein transferase and, thereby, of farnesylation of the Ras protein, blocks the ability of Ras to transform normal cells to cancer cells. Surprisingly, the compounds of the invention inhibit Ras farnesylation and, thereby, generate soluble Ras which, as indicated *infra*, can act as a dominant negative inhibitor of Ras function. While soluble Ras in cancer cells can become a dominant negative inhibitor, soluble Ras in normal cells would not be an inhibitor.

A cytosol-localized (no Cys-Aaa¹-Aaa²-Xaa box membrane domain present) and activated (impaired GTPase activity, staying bound to GTP) form of Ras acts as a dominant negative Ras inhibitor of membrane-bound Ras function (Gibbs *et al.*, *Proc. Natl. Acad. Sci. USA* 86:6630-6634 (1989)). Cytosol-localized forms of Ras with normal GTPase activity do not act as inhibitors. Gibbs *et al.*, *ibid*, showed this effect in *Xenopus* oocytes and in mammalian cells.

Administration of compounds of the invention to block Ras farnesylation not only decreases the amount of Ras in the membrane but

- 4 -

also generates a cytosolic pool of Ras. In tumor cells having activated Ras, the cytosolic pool acts as another antagonist of membrane-bound Ras function. In normal cells having normal Ras, the cytosolic pool of Ras does not act as an antagonist. In the absence of complete inhibition of farnesylation, other farnesylated proteins are able to continue with their functions.

Farnesyl-protein transferase activity may be reduced or completely inhibited by adjusting the compound dose. Reduction of farnesyl-protein transferase enzyme activity by adjusting the compound dose would be useful for avoiding possible undesirable side effects such as interference with other metabolic processes which utilize the enzyme.

These compounds are inhibitors of farnesyl-protein transferase. Farnesyl-protein transferase utilizes farnesyl pyrophosphate to covalently modify the Cys thiol group of the Ras CAAX box with a farnesyl group. Inhibition of farnesyl pyrophosphate biosynthesis by inhibiting HMG-CoA reductase blocks Ras membrane localization *in vivo* and inhibits Ras function. Inhibition of farnesyl-protein transferase is more specific and is attended by fewer side effects than is the case for a general inhibitor of isoprene biosynthesis.

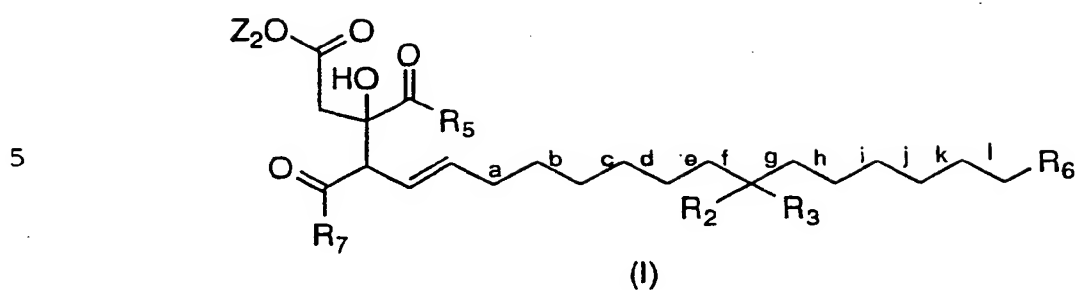
Previously, it has been demonstrated that tetrapeptides with the CAAX sequence inhibit Ras farnesylation (Schaber *et al.*, *ibid*: Reiss *et al.*, *ibid*; Reiss *et al.*, *PNAS*, 88:732-736 (1991)). However, the reported inhibitors of farnesyl-transferase are metabolically unstable or inactive in cells.

Pharmaceutical compositions containing the compounds of this invention and methods of treatment utilizing these compositions for use in inhibiting farnesyl-protein transferase and farnesylation of the oncogene protein Ras are described herein.

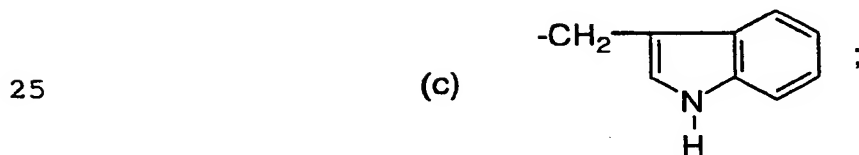
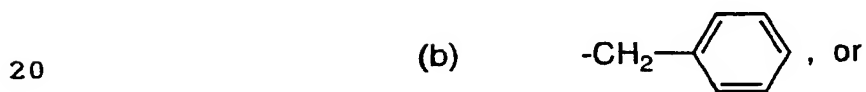
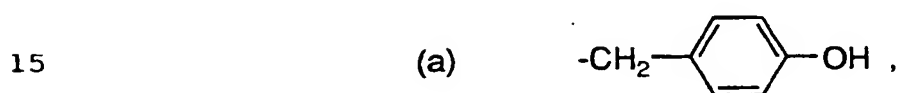
DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to novel compounds of structural formula (I) which are squalene synthase inhibitors, antihypercholesterolemic agents, antifungal agents, and anticancer agents:

- 5 -



10 wherein
R₁ is:



30 R₂ and R₃ are each H, or R₂ is H and R₃ is OH, or R₂ and R₃ together are oxo;
R₄ is H and R₅ is OZ₃, or R₄ and R₅ together are a bond to the nitrogen, forming a 2,5-dioxopyrrolidine ring;

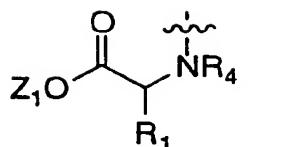
- 6 -

R₆ is

- (a) hydrogen, or
- (b) C₁₋₃ alkyl;

R₇ is:

- (a) -OZ₁, or
- (b)



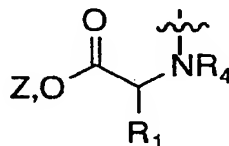
each of a, b, c, d, e, f, g, h, i, j, k, and l are a single bond or one of a, b, c, d, e, f, g, h, i, j, k, and l is a double bond, provided that when f or g is a double bond, R₂ is absent and R₃ is H; and

Z₁, Z₂ and Z₃ are independently:

- (a) H,
- (b) C₁₋₅alkyl, or
- (c) C₁₋₅alkyl substituted with
 - (i) phenyl, or
 - (ii) phenyl substituted with methyl, methoxy, halogen (Cl, Br, F, I) or hydroxy;

or a pharmaceutically acceptable salt of a compound of formula (I) provided that when

R₂ and R₃ are together oxo,
 R₄ is H,
 R₅ is OZ₃,
 R₆ is methyl and
 R₇ is



- 7 -

one of a, b, c, d, e, f, g, h, i, j, k, and l is a double bond.

5 A subclass is those compounds in which R₁ is -CH₂-3-indolyl and R₂ is H, R₃ is hydroxyl, R₄ is hydrogen, R₅ is OZ₃, and R₆ is methyl. Exemplifying this subclass are the compounds wherein Z₁, Z₂ and Z₃ are each hydrogen or a pharmaceutically acceptable salt thereof. The compound wherein Z₁, Z₂ and Z₃ are each hydrogen is hereafter referred to as Compound F. Further illustrating this subclass are those compounds in which one or more of Z₁, Z₂ or Z₃ is C₁-5
10 alkyl or C₁-5 alkyl substituted with phenyl or substituted phenyl wherein the substituent is methyl, methoxy, halogen or hydroxy.

Another subclass is those compounds in which R₁ is p-hydroxybenzyl, R₂ and R₃ together are oxo, R₄ is hydrogen, R₅ is OZ₃ and R₆ is n-propyl. Exemplifying this subclass are those compounds
15 wherein Z₁, Z₂ and Z₃ are each hydrogen or a pharmaceutically acceptable salt thereof. The compound wherein Z₁, Z₂ and Z₃ are each hydrogen is hereafter referred to as Compound G. Further illustrating this subclass are those compounds in which R₁ is p-hydroxybenzyl and R₂ and R₃ are together oxo, R₄ is hydrogen, R₅ is OZ₃, R₆ is n-propyl, and in which one or more of Z₁, Z₂ and Z₃ is C₁-5 alkyl or
20 C₁-5 alkyl substituted with phenyl or substituted phenyl wherein the substituent is methyl, methoxy, halogen or hydroxy.

In a third subclass are those compounds wherein R₁ is p-hydroxybenzyl, R₂ and R₃ are together oxo, R₄ and R₅ are together a
25 single bond and R₆ is methyl. Exemplifying this subclass are those compounds wherein Z₁ and Z₂ are each hydrogen or a pharmaceutically acceptable salt thereof. The compound of this subclass wherein Z₁ and Z₂ are each hydrogen is hereafter referred to as Compound H. Further illustrating this subclass are those compounds in which one or
30 both of Z₁ and Z₂ is C₁-5 alkyl or C₁-5 alkyl substituted with phenyl or substituted phenyl wherein the substituent is methyl, methoxy, halogen or hydroxy.

- 8 -

In a fourth subclass are those compounds wherein R₁ is p-hydroxybenzyl, R₂ and R₃ are each hydrogen, R₅ is -OZ₃ and R₆ is methyl. Exemplifying this subclass are those compounds wherein Z₁, Z₂ and Z₃ are each hydrogen or a pharmaceutically acceptable salt thereof. The compound of this subclass wherein Z₁, Z₂ and Z₃ are each hydrogen is hereafter referred to as Compound E. Further illustrating this subclass are those compounds in which one or more of Z₁, Z₂ and Z₃ is C₁-5 alkyl or substituted C₁-5 alkyl substituted with phenyl or substituted phenyl wherein the substituent is methyl, methoxy, halogen or hydroxy.

Additional compounds of the first class of compounds of this invention wherein Z₁ is OH, Z₂ is OH, Z₃ is OH, R₄ is H, R₅ is OH, and R₁, R₂, R₃, and R₆ are as listed in Table 1 below:

TABLE 1

Compound	R ¹	R ²	R ³	R ⁶
L	benzyl	-H	-H	-CH ₃
M	benzyl	oxo with R ₃	oxo with R ₂	-CH ₂ CH ₂ CH ₃
N	p-hydroxybenzyl	-H	-OH	-CH ₃
O	p-hydroxybenzyl	oxo with R ₃	oxo with R ₂	-H
R	benzyl	oxo with R ₃	oxo with R ₂	-H
S	benzyl	-H	-OH	-CH ₃

In a second class of compounds of this invention, R₇ is -N(R₄)CH(R₁)C(=O)OZ₁, and one of a, b, c, d, e, f, g, h, i, j, k, and l is a double bond provided that when f or g is a double bond, R₂ is absent.

- 9 -

One sub-class of this second class of compounds is those compounds in which R₁ is benzyl, R₂ and R₃ are each H, R₄ is hydrogen, R₅ is OZ₃, and R₆ is methyl. Exemplifying this sub-class are those compounds wherein Z₁, Z₂ and Z₃ are each hydrogen or a pharmaceutically acceptable salt thereof. The compound wherein Z₁, Z₂ and Z₃ are each hydrogen is hereafter referred to as Compound Q. Further illustrating this sub-class are those compounds in which one or more of Z₁, Z₂ or Z₃ is C₁-5alkyl or C₁-5alkyl substituted with phenyl or substituted phenyl wherein the substituent is methyl, methoxy, halogen or hydroxy.

Another subclass are those compounds in which R₁ is p-hydroxybenzyl, R₂ and R₃ are together oxo, R₄ is hydrogen, R₅ is -OZ₃, R₆ is methyl and one of a, b, c, d, e, h, i, j, k, and l is a double bond. Exemplifying this subclass are those compounds wherein Z₁, Z₂ and Z₃ are each hydrogen or a pharmaceutically acceptable salt thereof. The compound of this second class wherein R₁ is p-hydroxybenzyl, R₂ and R₃ are together oxo, R₄ is hydrogen, R₅ is OZ₃, R₆ is methyl and Z₁, Z₂ and Z₃ are each hydrogen is hereafter referred to as Compound P. Further illustrating this subclass are those compounds in which R₁ is benzyl and in which one or more of Z₁, Z₂ or Z₃ is C₁-5 alkyl or C₁-5 alkyl substituted with phenyl or substituted phenyl wherein the substituent is methyl, methoxy, halogen or hydroxy.

In a third class of compounds of the present invention, R₇ is -OZ₁. In one particular subclass of this third class of the invention, R₂ and R₃ are both H, R₅ is -OZ₃, R₆ is methyl. The compound in this subclass wherein Z₁, Z₂ and Z₃ are each hydrogen and each of a, b, c, d, e, f, g, h, i, j, k, and l are a single bond is hereafter referred to as Compound T.

The compounds of formula (I) are prepared in an aerobic fermentation procedure employing a novel fungal culture, MF5628, identified as Trichoderma viride Pers. or a mutant thereof. A mutant refers to an organism in which some gene on the genome is modified, leaving functional and heritable the gene or genes responsible for the organism's ability to produce the compounds of formula (I) in

- 10 -

recoverable amounts. Trichoderma viride is a common and geographically widespread organism, present in many soils and organic substrata throughout the world.

5 The culture MF5628 is that of a fungus, Trichoderma viride Pers., isolated from soil collected in Kolonia, Pohnpei (formerly Ascension Island), Federated States of Micronesia. This culture has been deposited with the American Type Culture Collection at 12301 Parklawn Drive, Rockville, MD 20852 as ATCC 74084 on July 30, 1991, under the conditions of the Budapest Treaty.

10 Within the genus Trichoderma, this strain can be assigned to Trichoderma viride species aggregate (Rifai, M. A. 1969. A revision of the genus Trichoderma. CMI Mycological Paper 116: 1-56) because it lacks sterile terminal appendages on the conidiophores, the phialides are long, tapered and arranged in a regular, dendritic pattern, and the
15 conidia are, for the most part, subglobose and roughened.

The culture MF5628, identified as Trichoderma viride exhibits the following morphological features.

Colonies growing rapidly on most standard mycological media. On corn meal agar, malt-extract agar, or oatmeal agar reaching
20 40-45 mm in 48 hours at 27°C and covering a 9 cm plate in about five days. At first, colonies on malt-extract agar hyaline to white, sparse to loosely floccose, at maturity becoming dark yellowish green to dark green because of conidial production, Dark Green, Dark Yellowish Green, reverse olive yellow to Light Yellowish Olive (capitalized color
25 names from Ridgway, R., 1912. Color Standards and Nomenclature, Washington, D.C.). On corn meal agar, tufts of conidiophores forming concentric rings, alternating with zones of hyaline, hyphae, on malt-extract and oatmeal agars, conidiophores develop uniformly over the agar surface.

30 Mycelium hyaline, septate, smooth-walled, 2.6-3.8 µm wide, forming chlamydospores which are globose to subglobose, smooth, 5.7-9.5 µm diameter. Conidiophores arising from surface of colony, often aggregated in pulvinate conidial pustules, profusely branched at wide angles, either singly or in groups of two or three in

- 11 -

opposite or whorled arrangement, with branches decreasing in length towards the apex, terminating with phialides. Conidiogenous cells enteroblastic, phialidic, narrowly flask-shaped, slender, tapered at the apices, 5.7-9.5 x 2 μ m, straight or slightly curved, single, or in groups of two or three, at wide angles to the conidiophore branch, forming a regular dendritic pattern, never together in dense aggregations. Conidia subglobose to ovoid 3.0-4.5 x 2.6-3.0 μ m, pale green in KOH, slightly roughened, accumulating as a cluster at tips of the phialides.

Compounds of this invention can be obtained by culturing the above-noted microorganism in an aqueous nutrient medium containing sources of assimilable carbon and nitrogen, preferably under aerobic conditions. Nutrient media may also contain mineral salts and defoaming agents.

The preferred sources of carbon in the nutrient medium are carbohydrates such as glucose, glycerin, starch, dextrin, and the like. Other sources which may be included are maltose, mannose, sucrose, and the like. In addition, complex nutrient sources such as oat flour, corn meal, millet, corn and the like may supply utilizable carbon. The exact quantity of the carbon source which is used in the medium will depend, in part, upon the other ingredients in the medium, but is usually found in an amount ranging between 0.5 and 5 percent by weight. These carbon sources can be used individually in a given medium or several sources in combination in the same medium.

The preferred sources of nitrogen are amino acids such as glycine, methionine, proline, threonine and the like, as well as complex sources such as yeast extracts (hydrolysates, autolysates), dried yeast, tomato paste, soybean meal, peptone, corn steep liquor, distillers solubles, malt extracts and the like. Inorganic nitrogen sources such as ammonium salts (eg. ammonium nitrate, ammonium sulfate, ammonium phosphate, etc.) can also be used. The various sources of nitrogen can be used alone or in combination in amounts ranging between 0.2 to 70 percent by weight of the medium.

The carbon and nitrogen sources are generally employed in combination, but need not be in pure form. Less pure materials which

- 12 -

contain traces of growth factors, vitamins, and mineral nutrients may also be used. Mineral salts may also be added to the medium such as (but not limited to) calcium carbonate, sodium or potassium phosphate, sodium or potassium chloride, magnesium salts, copper salts, cobalt salts and the like. Also included are trace metals such as manganese, iron, molybdenum, zinc, and the like. In addition, if necessary, a defoaming agent such as polyethylene glycol or silicone may be added, especially if the culture medium foams seriously.

The preferred process for production of compounds of this invention consists of inoculating spores or mycelia of the producing organism into a suitable medium and then cultivating under aerobic conditions.

Compounds of formula (I) may be isolated from the aerobic fermentation of a culture of MF5628 (ATCC 74084). A culture of MF5628 (ATCC 74084) is defined as substantially free of its natural soil contaminants and capable of forming compounds of structural formula (I) in recoverable amounts. A biologically pure culture of MF5628 (ATCC 74084) may also be employed. A biologically pure culture is substantially free from viable deleterious contaminating microorganisms.

The fermentation procedure generally is to first inoculate a preserved source of culture into a nutrient seed medium and to obtain, sometimes through a two-step process, growth of the organisms which serve as seeds in the production of the active compounds. After inoculation, the flasks are incubated with agitation at temperatures ranging from 20 to 30°C, preferably 25 to 28°C. Agitation rates may range up to 400 rpm, preferably 200 to 220 rpm. Seed flasks are incubated over a period of 2 to 10 days, preferably 2 to 4 days. When growth is plentiful, usually 2 to 4 days, the culture may be used to inoculate production medium flasks. A second stage seed growth may be employed, particularly when going into larger vessels. When this is done, a portion of the culture growth is used to inoculate a second seed flask incubated under similar conditions but employing shorter time.

- 13 -

After inoculation, the fermentation production medium is incubated for 3 to 30 days, preferably 6 to 22 days, with or without agitation (depending on whether liquid or solid fermentation media are employed). The fermentation is conducted aerobically at temperatures ranging from 20 to 40°C. If used, agitation may be at a rate of 200 to 400 rpm. To obtain optimum results, the temperatures are in the range of 22 to 28°C, most preferably 24 to 26°C. The pH of the nutrient medium suitable for producing the active compounds is in the range of 3.5 to 8.5, most preferably 5.0 to 7.5. After the appropriate period for production of the desired compound, fermentation flasks are harvested and the active compound isolated.

An alcoholic or oxygenated solvent such as an ester or ketone, preferably MEK is stirred with the solid fermentation, and the MEK extract is collected by vacuum filtration. The solids remaining can be extracted and the extracts combined and concentrated in vacuo. The concentrated extract is dissolved in a pH 4.5 buffer, preferably 60 parts acetonitrile to 40 parts 50 mM sodium formate pH 4.5. The solution is extracted with a highly hydrophobic solvent such as hexanes to yield a defatted extract. The defatted aqueous extract is purified by anion exchange chromatography preferably BIORAD AG4X4 (formate cycle), washing with the aqueous buffer and eluting. Fractions containing the mixture of the compounds of the invention are pooled, diluted with water and extracted into an organic solvent such as ethyl acetate. The extract is washed, dried and concentrated in vacuo.

Further purification is accomplished using reversed-phase high pressure liquid chromatography (RP-HPLC). The preferred adsorbent for this chromatography is a octadecylsilane bonded phase silica gel. The preferred eluant for RP-HPLC is a mixture of acetonitrile and water buffered at low pH, such as 0.1% phosphoric or trifluoroacetic acid.

Further purification may also be accomplished using high speed countercurrent chromatography. The preferred solvent system is a mixture of hexanes: ethyl acetate: methanol: 0.1% aqueous phosphoric acid (5:5:5:5). The preferred apparatus is the ITO multilayer-coil

- 14 -

manufactured by P.C. Inc., Potomac, Maryland, USA; however, other countercurrent chromatography equipment may be used.

5 Esters of Compounds E, F, G, H, L, M, N, O, P, Q, R, S and T may be prepared by dissolving a compound selected from Compound E, F, G, H, L, M, N, O, P, Q, R, S, and T in a dry organic solvent, preferably tetrahydrofuran (THF) at 0-30°C and treating with the appropriately substituted isourea for 8-24 hours, cooling to -15°C and filtering the urea. The mono-, di- and tri- esters of Compounds E, F, G, H, L, M, N, O, P, Q, R, S and T may be prepared by varying the number of equivalents of isourea used. The filtrate is concentrated
10 under reduced pressure to yield the desired ester.

 The present invention is also directed to a method of inhibiting cholesterol biosynthesis which comprises the administration to a subject in need of such treatment a nontoxic therapeutically effective amount of a compound represented by structural formula (I) and
15 pharmaceutically acceptable salts thereof. Specifically, the compounds of this invention are useful as antihypercholesterolemic agents for the treatment of arteriosclerosis, hyperlipidemia, familial hypercholesterolemia and the like diseases in humans. They may be administered orally or parenterally in the form of a capsule, a tablet, an injectable preparation or the like. It is usually desirable to use the oral
20 route. Doses may be varied, depending on the age, severity, body weight and other conditions of human patients, but daily dosage for adults is within a range of from about 20 mg to 2000 mg (preferably 20 to 100 mg) which may be given in two to four divided doses. Higher
25 doses may be favorably employed as required.

 In addition, the present invention is directed to a method of inhibiting the enzyme squalene synthase which comprises the administration to a subject in need of such treatment a nontoxic therapeutically effective amount of a compound represented by
30 structural formula (I) and pharmaceutically acceptable salts thereof. Specifically, the compounds of this invention are useful as antihypercholesterolemic agents for the treatment of arteriosclerosis, hyperlipidemia, familial hypercholesterolemia and the like diseases in

- 15 -

humans. They may be administered orally or parenterally in the form of a capsule, a tablet, an injectable preparation or the like. It is usually desirable to use the oral route. Doses may be varied, depending on the age, severity, body weight and other conditions of human patients, but daily dosage for adults is within a range of from about 20 mg to 2000 mg (preferably 20 to 100 mg) which may be given in two to four divided doses. Higher doses may be favorably employed as required.

The pharmaceutically acceptable salts of the compounds of this invention include those formed from cations such as sodium, potassium, aluminum, calcium, lithium, magnesium, zinc, and from bases such as ammonia, ethylenediamine, N-methyl-glutamine, lysine, arginine, ornithine, choline, N,N'-dibenzylethylenediamine, chloro-procaine, diethanolamine, procaine, N-benzylphenethylamine, diethylamine, piperazine, tris(hydroxymethyl)aminomethane, and tetramethylammonium hydroxide. The salts included herein encompass those wherein one, two or all three of the carboxyl groups are in the salt form. These salts may be prepared by standard procedures.

The compounds of this invention may also be administered in combination with other cholesterol-lowering agents such as those which inhibit another enzyme in the biosynthetic pathway in the synthesis of cholesterol. Examples of such agents would include but are not limited to HMG-CoA reductase inhibitors, HMG-CoA synthase inhibitors, and squalene epoxidase inhibitors. Illustrative of such HMG-CoA reductase inhibitors are lovastatin, simvastatin, pravastatin and fluvastatin. Examples of HMG-CoA synthase inhibitors are the beta-lactone derivatives disclosed in U.S. Patents 4,806,564; 4,816,477; 4,847,271; and 4,751,237; the beta-lactam derivatives disclosed in U.S. 4,983,597 and U.S.S.N. 07/540,992 filed June 20, 1990; and the substituted oxacyclopropane analogues disclosed in European Patent Publication EP O 411 703. Illustrative examples of squalene epoxidase inhibitors are disclosed in European Patent Publication EP O 318 860 and in Japanese Patent Publication J02 169-571A. LDL-receptor gene inducer molecules are disclosed in U.S. Patent Application S.N. 07/670,640 filed March 18, 1991. Other cholesterol lowering agents

- 16 -

that may be administered include niacin, probucol, the fibric acids: clofibrate and gemfibrozil, and LDL-receptor gene inducers.

Representative of such combinations are those containing about 10-400 mg of a compound of formula (I) in combination with about 20-100 mg of an HMG-CoA reductase inhibitor, 20 to 200 mg of a HMG-CoA
5 synthase inhibitor, or 1 to 200 mg of a squalene epoxidase inhibitor, or 250-1000 mg of probucol, or 600-1200 mg of gemfibrozil, or 1-2 g of clofibrate, or 3-6 g of niacin, or 20-300 mg of an LDL-receptor gene inducer.

10 The compounds of this invention may also be co-administered with pharmaceutically acceptable non-toxic cationic polymers capable of binding bile acids in a non-resorbable form in the gastrointestinal tract. Examples of such polymers include cholestyramine, colestipol and poly[methyl-(3-tri-methyl)amino-propyl]imino-
15 trimethylene dihalide. The relative amounts for co-administration of the compounds of this invention and these polymers is between 1:100 and 1:15,000 (w/w).

The intrinsic squalene synthase inhibitory activity of representative compounds of this invention was measured by the
20 standard in vitro protocol described below:

PREPARATION OF HUMAN HepG2 CELL ENZYME

1. SOURCE: HEPG2 CELL LINE (Liver, hepatoblastoma, Human) ATCC No. HB 8065
25

2. CELL GROWTH AND MAINTENANCE

Culture Medium: Minimum essential medium (MEM) with non-essential amino acids, sodium pyruvate, and 10% fetal bovine serum. The medium was changed twice weekly. A confluent
30 monolayer was achieved in 1 week. The growth medium was prepared as listed below.

- 17 -

	<u>Solution</u>	<u>Volume (mL)</u>
	1. MEM (Gibco #320-1090AK) With Earle's salts and L-glutamine	1000
5	2. Penicillin (10,000 units/mL), streptomycin (10,000 mg/mL), Gibco #600-5140 PG	10
	3. MEM sodium pyruvate, 10 mM (100X) Gibco #320-1140	10
	4. MEM nonessential amino acids, 10 mM (100X) Gibco #320-1140AG	10
10	5. L-glutamine, 200 mM (100X), Gibco #320-5030AG	10
	6. Hyclone fetal bovine serum, defined, Hyclone #A-111-L	100

15 SUBCULTURE PROCEDURE: The medium was removed and washed with PBS (Phosphate-Buffered Saline 15.6 mM, pH 7.0). Fresh trypsin (0.25%)-EDTA (0.02%) with Hank's Balanced Salt solution was added and the flask was allowed to stand for a minute before the trypsin solution was removed. The flask was incubated at 20 37°C until cells detached. Fresh medium was added and the cells were dispersed and dispensed into new flasks. Subcultivation ratio: 1:6.

PREPARATION OF DELIPIDATED SERUM: Fetal calf serum (100 mL) and CAB-O-SIL (2 grams) were stirred overnight at 25 4°C and centrifuged at 16,000 rpm for 5 h. The supernatant was filtered and the serum was stored at 4°C.

48 h prior to harvest, cells grown in MEM with 10% Fetal Calf serum were switched to MEM with 10% delipidated serum.

30 HARVEST: The medium was removed and the cells were washed with PBS. Fresh trypsin (0.25%)-EDTA (0.02%) with Hanks' Balanced Salt solution was added and allowed to stand and removed. The flask was incubated at 37°C until the cells detached. MEM medium (6 mL/flask) was added to suspend cells and combined into a centrifuge tube. The cells were spun at 1,000 rpm for 5 mins. The cell pellet was

- 18 -

resuspended in PBS and recentrifuged. Cells were counted (2.5×10^9 yield from 18 flasks (75 cm^2)), and resuspended in 10 mL of 50mM HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid]) containing 5mM MgCl_2 , 2mM MnCl_2 , 10mM DTT, pH 7.5 (enzyme suspension buffer).

5 CELL EXTRACTS: The cell suspension was sonicated (probe sonicator setting #60, pulse) on ice for 2 min. After a 1 min. cooling on ice, the sonication was repeated until greater than 90% of the cells were broken as observed microscopically. The supernatant was
10 centrifuged for 10 mins. at 10,000 rpm and the supernatant was transferred to a clean tube and centrifuged at 20,000 rpm for 20 mins. The HepG2 enzyme preparation was centrifuged at 34,000 rpm to separate the cytosol and microsomal enzymes. The resulting pellet from the 34,000 rpm centrifugation, containing the squalene synthase, was
15 resuspended in 5 mL of enzyme suspension buffer. The enzyme suspension was diluted and used to perform the squalene synthase assay using $3 \mu\text{M}$ ^3H -farnesyl pyrophosphate as the substrate.

Preparation of Yeast Enzyme

20 S. cerevisiae W303-1A (MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1) was grown at 30°C for 24 hours in YPD medium (2% yeast extract, 1% bacto-peptone, 2% glucose) supplemented with $20 \mu\text{g/mL}$ adenine. The cells were washed and resuspended in a minimal volume of breakage buffer (100 mM KPO_4 ,
25 pH 7.4, 3 mM DTT, 1 mM PMSF). The cells were added to the small chamber of the Bead-Beater Cell Disrupter (Biospec Products, Bartlesville, OK) containing 100 g of 0.5 mm glass beads. Buffer was added to fill the chamber. The sealed chamber was packed in ice and processed for 4 cycles of 30 sec with 2 min. cooling between cycles. Cell breakage was determined to be $>90\%$ by phase-contrast
30 microscopy. The glass beads were removed by Whatman filter paper and the filtrate collected into an ice-immersed filter flask. The extract was centrifuged at $1500 \times g$ for 10 min at 4°C . The pellet (P1) containing unbroken cells and cell walls was discarded. The supernatant

- 19 -

(S1) was centrifuged at 30000 x g for 30 min at 4°C. The pellet (P2) contained about two-thirds of the total squalene synthase activity. The supernatant (S2) can be centrifuged at 100,000 x g for 60 min at 4°C to pellet the remaining activity (P3). The pellet fractions were suspended at 10-20 mg/ml protein in breakage buffer containing 25% glycerol. The activity was stable for several months at -80°C.

Squalene Synthase Assay

Reactions were performed in 1.2 mL polypropylene tube strips of eight. The standard reaction mixture in a total volume of 0.140 mL, contained HEPES-Na⁺ buffer pH 7.5 50 mM, NADPH 1 mM, MgCl₂ 5.5 mM, KF 11 mM, DTT 3 mM, terbinafine (Yeast squalene epoxidase inhibitor, Sandoz SF86-327) or mammalian squalene epoxidase inhibitor (Banyu FW-439H) 1 µg/mL, farnesyl pyrophosphate (FPP) 5 µM (NEN), ³H-farnesyl pyrophosphate 0.25 µCi (NEN, 20 Ci/mmmole), enzyme preparation 0.1 µg yeast protein or 0.4 µg HepG2 protein and test sample in 5 µl DMSO. All assays components except FPP/³H-FPP were pre-incubated for 12 min at 30°C to allow inhibitor to bind to the enzyme. The reactions were started with FPP/³H-FPP addition. After 12 min at 30°C, the reactions were stopped by addition of 0.2 mL ethanol. The reaction product was extracted with 0.4 mL heptane containing 0.4 µL carrier squalene 0.2 mL of the heptane extract was counted by liquid scintillation. The IC₅₀ was determined as the concentration of inhibitor which gave 50% of the control (no inhibitor) enzyme activity as determined from a logarithmic curve fit of the data from a drug titration.

Percent inhibition is calculated by the formula:

$$\frac{(\text{Control} - \text{Sample}) \times 100}{\text{Control} - \text{Blank}}$$

IC₅₀ values were determined by plotting the log of the concentration of the test compound versus the percentage inhibition.

- 20 -

The IC₅₀ is the concentration of inhibitor that gives 50% inhibition as determined from these plots.

Alternatively, the intrinsic squalene synthase inhibitory activity of the compounds formed in the fermentation of the microorganisms of the present invention may be measured by the standard in vitro protocol described below:

Preparation of Rat Liver Microsomes:

Male, CHARLES RIVER® CD rats (120 to 150 g) were fed a diet containing 0.1% lovastatin for 4 days. The livers from these rats were homogenized in 5 volumes (mL/g) of ice cold 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid), 5 mM EDTA(ethylenediaminetetraacetic acid) pH 7.5 with a Potter-Elvehjem type tissue grinder. The homogenate was centrifuged twice at 20,000 x g for 15 min. at 4°C, discarding the pellet each time. The supernatant was then centrifuged at 100,000 x g for 1 hr at 4°C. The resulting microsomal pellet was resuspended in a volume of the above homogenizing buffer equal to one-fifth the volume of the original homogenate. This microsomal preparation has a protein concentration of about 7 mg/mL. The microsomal suspensions were stored in aliquots at -70°C. Squalene synthase activity in these aliquots is stable for a least several months.

Partial Purification of Prenyl Transferase

Prenyl transferase was purified to use in the enzymatic synthesis of radiolabelled farnesyl pyrophosphate. Prenyl transferase was assayed by the method of Rilling (Methods in Enzymology 110, 125-129 (1985)) and a unit of activity is defined as the amount of enzyme that will produce 1 μ mole of farnesyl pyrophosphate per minute at 30°C in the standard assay.

The livers of 23 forty-day old male rats that had been fed 5% cholestyramine plus 0.1% lovastatin were homogenized in a Waring blender in 1 liter of 10 mM mercaptoethanol, 2 mM EDTA, 25 mM leupeptin, 0.005% phenylmethylsulfonyl fluoride, pH 7.0 containing 0.1

- 21 -

trypsin inhibitor units of aprotinin/mL. The homogenate was centrifuged at 20,000 x g for 20 min. The supernatant was adjusted to pH 5.5. with 6 N HOAc and centrifuged at 100,000 x g for 1 hour. This supernatant was adjusted to pH 7.0 with 3 N KOH and a 35-60% ammonium sulfate fraction taken. The 60% pellet was redissolved in 60 mL of 10 mM potassium phosphate, 10 mM mercaptoethanol, 1 mM EDTA pH 7.0 (Buffer A) and dialyzed against two 1 liter changes of Buffer A. This dialyzed fraction was applied to a 12.5 x 5 cm column of DEAE-sepharose 4B equilibrated with Buffer A. The column was washed with 700 mL of Buffer A and a 1 liter gradient from Buffer A to 100 mM potassium phosphate, 10 mM mercaptoethanol, 1 mM EDTA, pH 7.0. Fractions having a specific activity greater than 0.20 units/mg were combined, solid ammonium sulfate was added to bring to 60% saturation and pelleted. The pellet was dissolved in 8 mL 10 mM Tris, 10 mM β -mercaptoethanol pH 7.0 (Buffer B). The redissolved pellet was taken to 60% saturation with ammonium sulfate by adding 1.5 volumes of saturated ammonium sulfate in Buffer B. This ammonium sulfate suspension contained 3.5 units/mL with specific activity of 0.23 units/mg and was free of isopentenyl pyrophosphate isomerase activity. This ammonium sulfate suspension was used for the synthesis of [4- 14 C]farnesyl-pyrophosphate and its activity was stable stored at 4°C for a least 6 months.

Enzymatic Synthesis of [4- 14 C]farnesyl-pyrophosphate

The solvent (ethanol: 0.15 N NH_4OH , 1:1) was removed from 55 mCi of [4- 14 C]isopentenyl pyrophosphate(47.9 mCi/mmol) by rotary evaporation. Six hundred microliters of 100 mM Tris, 10 mM MgCl_2 , 4 mM dithiothreitol pH 7.5 was added and the solution was transferred to a 1.5 mL Eppendorf centrifuge tube. Geranyl-pyrophosphate, 250 mL of a 20 mM solution, and 50 mL of the ammonium sulfate suspension of prenyl transferase were added to initiate the reaction. This incubation contained 5 mmoles of geranyl pyrophosphate, 1.15 mmoles of isopentenyl pyrophosphate, 6 mmoles of MgCl_2 of 0.18 units of prenyl transferase in a volume of 900 mL. The

- 22 -

incubation was conducted at 37°C. During the incubation, the mix turned cloudy white as the newly formed magnesium complex of farnesyl pyrophosphate precipitated out of solution. The [4-¹⁴C]farnesyl pyrophosphate was collected by centrifugation for 3 minutes at 14,000 rpm in an Eppendorf centrifuge tube, the supernatant removed, and the pellet was dissolved in 1.0 mL of 50 mM HEPES, 5 mM EDTA, pH 7.5. The yield was 50.7 mCi (92%) of [4-¹⁴C]farnesyl pyrophosphate. The [4-¹⁴C]farnesyl pyrophosphate was stored in aliquots at -70°C.

Squalene Synthase Assay

Reactions were performed in 16 x 125 mm screw cap test tubes. A batch assay mix was prepared from the following solution:

	mL per assay
1. 250 mM HEPES pH 7.5	20
2. NaF 110 mM	10
3. MgCl ₂ 55 mM	10
4. Dithiothreitol 30 mM	10
5. NADPH 10 mM (made fresh)	10
6. [4- ¹⁴ C]farnesylpyrophosphate 47.9 mCi/mmol, and 0.025 mCi/3.0 mL	3.0
7. H ₂ O	24

This assay mix was degassed under a vacuum and flushed with N₂. Solutions of the squalene synthase inhibitors were prepared either in DMSO or MeOH and a 1:120 dilution of the microsomal protein was made with the original homogenizing buffer. For each reaction, 87 mL of the assay mix was taken with 3 mL of an inhibitor solution (DMSO or MeOH in the controls), warmed to 30°C in a water bath and then the reaction was initiated by the addition of 10 mL of the 1:120 dilution of microsomal protein (0.6 mg protein total in the assay). The reactions were stopped after 20 minutes by the addition of 100 mL

- 23 -

of a 1:1 mix of 40% KOH with 95% EtOH. The stopped mix was heated at 65°C for 30 min., and cooled. Ten mL of heptane was added and the mix was vortexed. Two g of activated alumina was then added, the mix vortexed again, the alumina allowed to settle and 5 mL of the heptane layer was removed. Ten mL of scintillation fluid was added to the heptane solution and radioactivity was determined by liquid scintillation counting.

Percent inhibition is calculated by the formula:

$$1 - \left(\frac{[\text{Sample} - \text{Blank}]}{[\text{Control} - \text{Blank}]} \right) \times 100$$

The present compounds also demonstrate broad spectrum antifungal activity as determined by broth dilution methods. The compounds are particularly active towards filamentous fungi and yeasts including Candida albicans and Ustilago zeae. The sensitivity of filamentous fungi and yeast was determined using inhibitor dilution assays in microtiter format. The compounds were dissolved in DMSO at 2 mg/mL and diluted in 100 µL of DMSO. Exponential phase Candida, Cryptococcus and Ustilago cells were diluted in fresh YNB/G (Difco Yeast Nitrogen Base supplemented with 2% glucose) and YCB/Glu (Difco yeast carbon base with 2 mM l-glutamic acid) such that the inoculum was 1 x 10⁴ cells/mL. Aspergillus spores were harvested from a well-sporulated Sabouraud Dextrose Agar slant in 0.4% Tween 80 and diluted into media to give an inoculum of 1 x 10³ spores/mL. The wells were filled with 150 µL of inoculated media. The final drug concentration tested ranged from 40 to 0.313 µg/mL. The microtiter dishes were incubated at 29°C for 20 to 48 hours. The minimum inhibitory concentration (MIC) is defined as the lowest concentration to prevent visible growth after incubation for 20 hours at 29°C for the yeasts and 24 to 48 hours at 29°C for the filamentous fungi. The minimum fungicidal concentration was defined as the lowest concentration of drug showing no growth. Representative of the

- 24 -

antifungal activity are the minimum fungicidal concentration and minimum inhibitory concentration data shown below.

		Minimum Fungicidal Concentration ($\mu\text{g/mL}$)				
		COMPOUND				
5	Organism	G	L	N	O	T
	<u>Candida albicans</u> MY1055	0.25	>32	0.5	>32	8
10	<u>Candida albicans</u> MY1028	>16	>32	32	32	>32
	<u>Candida albicans</u> MY1750	2	>32	8	>32	4
15	<u>C. guilliermondi</u> MY1019	1	>32	16	32	4
20	<u>C. parapsilosis</u> MY1010	1	>32	8	16	32
	<u>C. pseudotropicalis</u> MY2099	0.13	>32	4	1	16
25	<u>C. tropicalis</u> MY1012	16	>32	32	>32	32
	<u>C. neoformans</u> MY1051	1	>32	4	16	4
30	<u>C. neoformans</u> MY1146	1	>32	8	16	>32

- 25 -

Organism	G	L	N	O	T
<u>Cryptococcus</u> <u>neoformans</u> MY2061	1	>32	4	16	16
<u>Cryptococcus</u> <u>neoformans</u> MY2062	1	>32	4	4	>32
<u>S. cerevisiae</u> MY1976	>16	>32	32	32	>32
<u>A. flavus</u> MF383	1	>32	8	16	16
Minimum Inhibitory Concentration ($\mu\text{g/mL}$)					
Organism	G	L	N	O	T
<u>A. fumigatus</u> MF5668	2	>32	32	>32	16
<u>A. fumigatus</u> MF5669	2	>32	32	>32	16
<u>Aspergillus</u> <u>fumigatus</u> MF4839	2	>32	32	>32	8

Thus, the present invention is also directed to a method of inhibiting fungal growth which comprises the application to the area in which growth is to be controlled an antifungally effective amount of a compound of Formula (I). Additionally, the present invention is directed to a method of treating fungal infections which comprises the administration to an organism in need of such treatment a nontoxic therapeutically effective amount of a compound represented by the structural formula (I) and pharmaceutically acceptable salts thereof. Based on the above MIC and MFC data it is determined that generally

- 26 -

from 2 to about 20 mg/kg should be employed as a unit dosage in an antifungal treatment.

5 The compounds of this invention are adaptable to being utilized in various applications of antifungal compositions. In such use, compounds may be admixed with a biologically inert carrier, generally with the aid of a surface active dispersing agent, the nature of which would vary depending on whether the use is for the control of pathogens infecting mammals such as man, or birds or reptiles, or for control of fungi in agriculture such as in soil or plant parts, or for the control of fungi in inanimate objects.

10 In compositions for medical applications, the compounds may be admixed with a pharmaceutically acceptable carrier, the nature of which will vary depending whether the composition is to be topical, parenteral or oral.

15 If said application is to be topical, the drug may be formulated in conventional creams and ointments such as white petroleum, anhydrous lanolin, cetyl alcohol, cold cream, glyceryl monostearate, rose water and the like.

20 For parenteral applications, the compounds may be formulated in conventional parenteral solutions such as 0.85 percent sodium chloride or 5 percent dextrose in water, or other pharmaceutically acceptable compositions.

25 Compositions for oral administration may be prepared by intimately mixing the component drugs with any of the usual pharmaceutical media, including, for liquid preparations, liquid carriers such as water, glycols, oils, alcohols, and the like; and for solid preparations such as capsules and tablets, solid carriers such as starches, sugars, kaolin, ethyl cellulose, surface active dispersing agents, generally with lubricant such as calcium stearate, together with binders, disintegrating agents and the like.

30 These compositions are then administered in amounts sufficient to obtain the desired antifungal effect. For medical application, the method comprises administering to a subject in need of treatment a therapeutically effective antifungal amount of a compound

- 27 -

of Formula I. The appropriate doses will vary depending on age, severity, body weight and other conditions. For topical application the compositions are applied directly to the area where control is desired. For internal administration, the composition may be applied by injection or may be administered orally.

5 For non-medical application, the product of the present invention, either singly or as a mixture, may be employed in compositions in an inert-carrier which includes finely divided dry or liquid diluents, extenders, fillers, conditioners and excipients, including various clays, diatomaceous earth, talc, and the like, or water and
10 various organic liquids such as lower alkanols, for example ethanol and isopropanol, or kerosene, benzene, toluene and other petroleum distillate fractions or mixtures thereof.

These compositions may be employed by applying to the surface of or incorporating in the medium to be protected. For the
15 control of rice blast, tomato late blight, tomato early blight, wheat leaf rust, bean powdery mildew and tomato Fusarium wilt, the compositions may be applied directly to the plant in topical application or administered to the soil for systemic application. The method comprises
20 administering to the affected plant, soil or medium to be protected an antifungally effective amount of the compound of Formula I.

The compounds of Formula I also exhibit Farnesyl-transferase inhibition, as shown below:

FARNESYL-TRANSFERASE ASSAY

I. PREPARATION OF SOLUTIONS REQUIRED FOR THE ASSAY

i. 1.0 M MgCl₂ (MW = 203.3)

203.3 g MgCl₂ was dissolved in 1.0 L distilled water, and
30 filtered through a sterile filter and stored at 4°C.

ii. 1.1 M HEPES (MW = 238.3) and 55.5 mM MgCl₂

264.5 g of HEPES was added to 700 mL distilled water followed by 56 mL of 1.0 M MgCl₂. The pH was adjusted to 7.5 with

- 28 -

sodium hydroxide and the volume was brought to 1000 mL. The solution was filtered through a sterile filter and stored at 4°C.

iii. 0.5 M Dithiothreitol (DTT, MW = 154.24)

77.12 mg of DTT was dissolved in 1.0 mL of distilled water. A few drops of 10 N NaOH was added to get the DTT to go into solution. 0.5 M DTT was divided into 500 µL aliquots and stored at -20°C.

iv. Preparation of Assay Buffer (Prepare fresh daily)

100 µL of 0.5 M DTT was added to 900 µL of 1.1 M HEPES, pH 7.5 and 55.5 mM MgCl₂ to yield a 10X buffer consisting of 1.0 M HEPES, pH 7.5, 50 mM MgCl₂ and 50 mM DTT.

v. Preparation of unlabelled farnesyl pyrophosphate (FPP)

The unlabelled FPP (MW=433.3) was provided as a 1.8 mM (1 µg/1.28 mL) solution. This solution was diluted to 2 µM with water and prepared fresh weekly and stored at -20°C. Glass tubes were used when preparing the unlabelled FPP.

vi. Preparation of ³H-FPP

The ³H-FPP (NEN #NET-1042, MW=433.3) was used directly from the bottle as provided by NEN. The stock concentration of the ³H-FPP was 25 µM.

vii. Preparation of the reaction mix for Totals and unknown samples (on ice)

The reaction mix was prepared as below:

Reagent	Stock Conc.	Vol to use/assay	Conc. in 20 µL	Final Conc. in assay
³ H-FPP	25 µM	0.2 µL	0.25 µM	50 nM
Cold-FPP	2000 nM	11.25 µL	1.125 µM	225 nM
ras-CVLS	1 mg/mL (47.62 µM)	6.3 µL	15 µM	3 µM
d-H ₂ O		2.25 µL		
TOTAL VOLUME		20 µL		

- 29 -

A Blank reaction mix was also prepared with the negative control ras-protein, and the Total/Sample reaction mix received the ras-CVLS. The same volumes were used to prepare the Blank reaction mix as those in Table 1.

viii. Preparation of the Farnesyl Transferase solution

The farnesyl transferase solution was prepared as shown below. The farnesyl transferase mix was prepared right before use.

Reagent	Stock Conc.	Vol to use/assay
d-H ₂ O		14.0 μ L
Buffer	stock = 10X	10.0 μ L
F.Transferase	stock = 1 mg/mL	1.0 μ L
Total Volume		25 μL

ix. Preparation of 1.0 M HCl in 100% Ethanol

The stop solution was prepared by mixing 43 mL of concentrated HCl (11.6 M) with 457 mL of 100% ethanol to yield 1.0 M HCl in ethanol.

V. STEPS FOR THE ASSAY

- i. The assay volume was 100 μ L, the volume of sample to be tested was 5 μ L and the time of incubation is 60 min at room temperature.
- ii. The TECAN 8000/505, an automatic pipetting station, added 50 μ L of water plus 5 μ L of sample to the assay tubes.
- iii. The ras-CVLS reaction mix (20 μ L) was manually added to totals and unknowns. The negative control ras-protein reaction mix (20 μ L) was manually added to the blank tubes.

- 30 -

- iv. The farnesyl transferase mix (25 μ L) was manually added to all tubes to initiate the reaction and the assay tubes were kept at room temperature for 60 min.
- v. The assay was stopped placing the assay tubes in an ice bath for 5 minutes.
- 5 vi. 1.0 mL of 1.0 M HCl in 100% ethanol was then added to each tube. The assay tubes (uncapped) were incubated at 37°C for 60 min. to facilitate the hydrolysis of the FPP.
- 10 vii. Assay tubes were harvested through a TOMTEC 96-well harvester (6 x 16 with extended tips, or SKATRON cell harvester) onto LKB double thickness filter mats.
- viii. The cell harvester was adjusted so that each well was washed with 10 mL of 100% ethanol.
- 15 ix. Filter mats were baked in microwave oven for 6-8 min. to dry the filtermats.
- x. The filter mats were placed in counting bags, 30 mL of LKB β -scint cocktail was added and the filter mats were counted for 120 sec. in the LKB β -plate counter.

20 VI. RESULTS AND DISCUSSION

Percent inhibition was calculated according to the following equation:

$$\% \text{ Inhibition} = \frac{[\text{Total} - \text{Sample}]}{[\text{Total} - \text{Blank}]} \times 100$$

25 The pharmaceutical compositions containing the compounds of structural formula (I) inhibit farnesyl-protein transferase and the farnesylation of the oncogene protein Ras. These compounds are useful as pharmaceutical agents for mammals, especially for humans. These
30 compounds may be administered to patients for use in the treatment of cancer. Examples of the type of cancer which may be treated with the compounds of this invention include, but are not limited to, colorectal carcinoma, exocrine pancreatic carcinoma, and myeloid leukemias.

- 31 -

The compounds of this invention may be administered to mammals, preferably humans, either alone, or preferably, in combination with pharmaceutically-acceptable carriers or diluents, optionally with known adjuvants, such as alum, in a pharmaceutical composition, according to standard pharmaceutical practice. The compounds can be administered orally or parenterally, including intravenous, intramuscular, intraperitoneal, subcutaneous and topical administration.

For oral use of a chemotherapeutic compound according to this invention, the selected compounds may be administered, for example, in the form of tablets or capsules, or as an aqueous solution or suspension. In the case of tablets for oral use, carriers which are commonly used include lactose and corn starch, and lubricating agents, such as magnesium stearate, are commonly added. For oral administration in capsule form, useful diluents include lactose and dried corn starch. When aqueous suspensions are required for oral use, the active ingredient is combined with emulsifying and suspending agents. If desired, certain sweetening and/or flavoring agents may be added. For intramuscular, intraperitoneal, subcutaneous and intravenous use, sterile solutions of the active ingredient are usually prepared, and the pH of the solutions should be suitably adjusted and buffered. For intravenous use, the total concentration of solutes should be controlled in order to render the preparation isotonic.

The present invention also encompasses a method of the treatment of cancer, comprising the administration of a pharmaceutical composition comprising a therapeutically effective amount of the compounds of this invention, with or without pharmaceutically acceptable carriers or diluents.

Suitable compositions of this invention include aqueous solutions comprising compounds of this invention and pharmacologically acceptable carriers, e.g. saline, at a pH level, e.g., 7.4. The solutions may be introduced into a patient's intramuscular blood-stream by local bolus injection.

- 32 -

When a compound according to this invention is administered into a human subject, the daily dosage will normally be determined by the prescribing physician with the dosage generally varying according to the age, weight, and response of the individual patient, as well as the severity of the patient's symptoms.

In one exemplary application, a suitable amount of compound is administered to a human patient undergoing treatment for cancer. Administration occurs in a amount between about 0.1 mg/kg of body weight to about 20 mg/kg of body weight of a mammal per day, preferably of between 0.5 mg/kg of body weight to about 10 mg/kg of body weight of a mammal per day.

The following examples illustrate the preparation of the compounds of formula (I) and their incorporation into pharmaceutical compositions and, as such, are not to be considered as limiting the invention set forth in the claims appended hereto.

The composition of media employed in the following Examples are listed below:

KF SEED MEDIUMTrace Element Mix #2

	<u>per liter</u>		<u>g/L</u>
20 Corn Steep Liquor	5 g	FeSO ₄ •7H ₂ O	1.0
Tomato Paste	40 g	MnSO ₄ •4H ₂ O	1.0
Oat Flour	10 g	CuCl ₂ •2H ₂ O	0.025
Cerelose	10 g	CaCl ₂ •2H ₂ O	0.1
25 Trace Element		H ₃ BO ₃	0.056
Mix #2	10 mL	(NH ₄) ₆ Mo ₇ O ₂₄ •4H ₂ O	0.019
Distilled Water	1000 mL	ZnSO ₄ •7H ₂ O	0.2
pH adjusted to 6.8		dissolved in 1L 0.6 N HCl	
(presterile)			
30 50 mL/non-baffled 250 mL			
Erlenmeyer flask			
autoclave 20 minutes (121°C,			
15 psi)			

- 33 -

Solid Substrate Production Medium

Solid substrate media prepared in nonbaffled 250 mL
Erlenmeyer flasks

5 BRF: Brown rice 5.0 g/flask
 Base liquid #3 20.0 mL/flask

Base liquid #3 g/L
 Yeast extract 1.0
 Sodium tartrate 0.5
10 KH₂PO₄ 0.5
 distilled water 1000.0 mL
 (no pH adjustment)
 autoclave 15 minutes (121°C, 15 psi)
 add 15.0 mL distilled water per flask
15 autoclave 20 minutes (121°C, 15 psi)

EXAMPLE 1

20 Preparation of Compound F

 A. Culturing MF5628

 Culture MF5628 was inoculated into 3 KF seed medium
 flasks using a mixture of hyphae and spores preserved in sterile soil.
25 The KF seed flasks were incubated for 74 hours at 25°C, 220 rpm, 85%
 humidity. At the end of this incubation, culture growth from the 3 seed
 flasks was pooled and then 2.0 mL aliquots were aseptically transferred
 to each of 50 BRF solid production medium flasks. These production
 flasks were then incubated statically at 25°C, 85% humidity for 21 days.
30 At harvest 50 mL methyl ethyl ketone (MEK) was added to each BRF
 production flask and the solid growth was manually broken apart into
 smaller pieces. Solvent treated flasks were returned to the gyrotory
 shaker for agitation at 220 rpm for 30 minutes in order to further break
 apart the mycelial mass as well as to improve contact of the solvent with

- 34 -

the cells. After shaking, the contents of these flasks were pooled by pouring the entire contents of the flasks (solids and all) into a 2L flask.

B. Isolation of Compound F

5 The 21-day 2000 mL solid fermentation in BRF media from above was extracted with 2500 mL of methyl ethyl ketone (MEK) by stirring an additional 1.5 hours at room temperatures. The extracted fermentation was then filtered through CELITE®.

10 A portion of the MEK extract (1500 mL) was concentrated to dryness in vacuo. The resulting residue was partitioned between hexane and methanol. The methanol layer was concentrated to dryness in vacuo. The residue was dissolved in 6:4 CH₃CN:50 mM NaOAc pH 4.5 and again extracted with hexanes. The aqueous methanol layer was then loaded at 150 mL/hour onto a 40 mL column of BIO-RAD AG4-X4 (acetate) which had been equilibrated with 6:4 CH₃CN:50 mM
15 NaOAc pH 4.5 (equilibration buffer). The column was then washed with 150 mL of equilibration buffer and then with 625 mL of a solution of 600 mL CH₃CN/44 mL conc. H₂SO₄/ 400 mL H₂O, collecting 25 mL fractions. Fractions 14 to 22 containing crude compound F were
20 pooled and extracted with ethyl acetate to yield 1.94 g of crude compounds. A portion (0.95 g) of this mixture was separated by high speed countercurrent chromatography using the apparatus manufactured by P.C. Inc. (11805 Kim Place, Potomac, Maryland, U.S.A.). The crude mixture was concentrated in vacuo to dryness and dissolved in 4
25 mL of the upper phase and 4 mL of the lower phase of a solvent system consisting of 5 parts hexanes: 5 parts ethyl acetate: 5 parts methanol: 5 parts 0.1% aqueous H₃PO₄. The sample was applied to the tail of a #10 preparative multilayer coil (P.C. Inc.) which had been filled completely with the lower phase of the above solvent system. The coil was then
30 eluted with the upper phase of the above solvent from the tail of the column to the head of the column at 3.0 mL/min., at a rotation speed of 800 rpm in the forward direction. After collection of 156 fractions (7.5 mL each), the elution of the column with the upper phase was stopped and the stationary phase eluted in the reverse direction of flow

- 35 -

(head to tail) at 9.0 mL/min collecting 1.0 min fractions to give fractions 1A to 39A. Fractions 28A - 39A from the high speed countercurrent chromatography were pooled and extracted once with ethyl acetate. The ethyl acetate layer was dried over anhydrous Na₂SO₄ and concentrated to yield crude Compound F. A portion of this mixture separated by column chromatography on PHENOMENEX ULTRACARB 5 ODS 30 (10 mm x 15 cm). The column was eluted at 3.0 mL/min. at 40°C with 90% A/10% B from time 0 min. to 5 min. followed by a linear gradient to 100% B at time 40 min. (A = 40% CH₃CN/60% H₂O/0.1% H₃PO₄, B = 75% CH₃CN/25% H₂O/0.1% H₃PO₄) collecting 1.0 min. fractions. Fraction 29 was extracted once with 3 mL CH₂Cl₂ and the CH₂Cl₂ layer removed and concentrated to dryness to yield Compound F.

EXAMPLE 2

Preparation of Compound G

A. Culturing MF5628

Culture MF5628 was grown according to the procedures of Example 1, Step A.

B. Isolation of Compound G

The isolation of Compound G was performed as described above for Compound F. Fraction 32 was extracted once with 3 ml CH₂Cl₂ and the CH₂Cl₂ layer removed and concentrated to dryness to yield Compound G.

EXAMPLE 3

Preparation of Compound H

A. Culturing MF5628

- 36 -

Culture MF5628 was grown according to the procedures of Example 1, Step A.

B. Isolation of Compound H

5 The isolation of Compound H was performed as described above in Example 1, Step B. Fractions 51 - 61 from the high speed countercurrent chromatography were pooled and concentrated to dryness to yield crude Compound H. A portion (20 mg) of this mixture was further purified by column chromatography on PHENOMENEX
10 ULTRACARB 5 ODS 30 (10 mm x 15 cm). The sample was dissolved in 0.26 mL mobile phase and loaded onto the column. The column was eluted at 40°C with a solution of 50% CH₃CN/50% H₂O/0.1% H₃PO₄ at 3.0 mL/min collecting 1.0 min. fractions. Fractions 6 - 7 were
15 pooled and extracted once with an equal volume of ethyl acetate. The ethyl acetate layer was dried over anhydrous Na₂SO₄ and concentrated to dryness to yield Compound H.

EXAMPLE 4-10

20 Step A: Isolation of a Crude Mixture of Compounds E, F, G, H, L, M, N, O, P, Q, R, S, and T

A 9 day 4230 mL solid fermentation in BRF media (grown according to the procedures of Example 1, Step A) was extracted with 4230 mL methyl ethyl ketone (MEK) by stirring 1 hour. The MEK
25 extract was collected by vacuum filtration and the solids extracted with an additional 3000 mL MEK. The second extract was collected by filtration, combined with the first extract and concentrated to dryness in vacuo to a greenish-brown syrup.

30 The concentrated extract from above was dissolved in 1500 mL of a solution of buffer 1 (800 mL water/1200 mL acetonitrile/4.28 mL concentrated formic acid and the pH adjusted 4.5 with a solution of 2.0 N NaOH). After complete dissolution the extract solution was adjusted to pH 4.5. This solution was then extracted twice with 1000 mL hexanes to yield a defatted extract (985 mL). A 940 mL portion of

- 37 -

the defatted aqueous extract was then applied at a flow rate of 650 mL/hour to a column of BioRad AG4X4 (formate cycle, 5.0 cm x 25 cm, 100 - 200 mesh) which had been prepared as follows: AG4X4 resin (free base form) was slurried in two volumes of a solution of 60 parts acetonitrile/40 parts water and the pH of the slurry adjusted to 4.5 with 2.0 N NaOH, the resin packed into a column of the above dimensions and then washed with 150 mL of buffer 1. After complete loading of the extract the column was washed with 300 mL buffer 1. The compounds were eluted from the resin with a solution of 1200 mL acetonitrile/800 mL water/11.2 mL concentrated sulfuric acid at a flow rate of 650 mL/hour and 150 mL fractions collected. Fractions 6-8 contained a mixture of the Compounds and were pooled (450 mL), 400 mL water added and the solution extracted with 800 mL of ethyl acetate. The ethyl acetate layer was removed, washed once with brine, dried over anhydrous Na₂SO₄ and concentrated in vacuo to a greenish-brown syrup.

Step B: Separation of Compounds E, F, G, H, L, M, N, O, P, Q, R, S, and T

The concentrated mixture of Compounds E, F, G, H, L, M, N, O, P, Q, R, S, and T from above was dissolved in ethyl acetate to a final volume of 50 mL. A 20 mL portion of this solution was concentrated to dryness in vacuo and separated by chromatography of a 5 cm x 25 cm column of 12 micron LICHROSPHER 60 RP - select B (E. MERCK). The column was equilibrated a 8 mL/min. with a mixture of 75% solution A (40 parts acetonitrile/60 parts water/0.1 part concentrated phosphoric acid) : 25% solution B (80 parts acetonitrile/20 parts water/0.1 part concentrated phosphoric acid). The 20 mL portion of the crude mixture of the Compounds was dissolved in solution B to a final volume of 5 mL and applied to the column. The column was eluted with a gradient of solution A:solution B as follows:

<u>Time (min)</u>	<u>% A / % B</u>	<u>Flow Rate (ml/min)</u>
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- 38 -

0	75/25	8
1	75/25	40
19	75/25	40
70	0/100	40

5 The column effluent was monitored at 220 nm and 0.5 min (20 mL) fractions were collected.

Fractions were analyzed by RP HPLC on PHENOMENEX ULTRACARB 5 ODS 30, 15 cm x 4.6 mm, flow rate 1.0 mL/min at 40°C, detection at 205 or 220 nm. Samples were dissolved 200 µg/mL in mobile phase and 20 µL injected. The following solvent systems were used:

Solvent System	CH ₃ CN	H ₂ O	H ₃ PO ₄
15 A	80%	20%	0.1%
B	52%	48%	0.1%
C	44%	56%	0.1%

20 Relative retention times (RTT) were measured with respect to Compound E for solvent system A.

Fractions 20,22,24,26 and 32 were individually desalted by extraction with an equal volume of ethyl acetate, the ethyl acetate washed with brine and then dried over anhydrous Na₂SO₄. The ethyl acetate layer was concentrated to yield crude fraction 20 (4 mg), fraction 22 (3 mg), fraction 24 (3.5 mg), fraction 26 (2 mg) and fraction 32 (9 mg). The above fractions and the fraction pools described below were analyzed by HR-FAB MS to characterize the minor components present.

30

EXAMPLE 4

Isolation of Compound E

- 39 -

Fractions 108-110 from the above reverse phase chromatography were pooled (volume = 140 mL), concentrated in vacuo to 41 mL and the solution extracted once with an equal volume of ethyl acetate. The ethyl acetate layer was washed once with brine, dried over anhydrous Na₂SO₄ and concentrated to dryness to yield
5 Compound E.

EXAMPLE 5

Isolation of Compounds F and Q

Fractions 74-77 from the above reverse phase chromatography were pooled, concentrated in vacuo and the solution extracted once with an equal volume of ethyl acetate. The ethyl acetate layer was washed once with brine, dried over anhydrous Na₂SO₄, and
15 concentrated to dryness to yield 24 mg of a mixture containing Compounds F and Q.

EXAMPLE 6

Isolation of Compound S and R

Fractions 78-79 from the above reverse phase chromatography were pooled, worked up as in Example 5 to yield 24 mg of a mixture containing Compounds S and R.

EXAMPLE 7

Isolation of Compounds L and T

Fractions 136-143 from the above reverse phase chromatography were pooled, worked up as described in Example 5 to
30 yield 9 mg of a mixture containing compounds L and T.

EXAMPLE 8

Isolation of Compound M

- 40 -

Fractions 111-119 from the above reverse phase chromatography were pooled, worked up as described in Example 5 to yield 19 mg of a mixture containing Compound M.

EXAMPLE 9

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Isolation of Compound N

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Fraction 48 from the above reverse phase chromatography was worked up as described in Example 5 to yield 54 mg of a mixture containing Compound N and Compound A.

EXAMPLE 10

Isolation of Compound O

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Fraction 44 from the above reverse phase chromatography was worked up as described in Example 5 to yield 5 mg of a mixture containing Compound O.

EXAMPLE 11

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Preparation of an Ammonium Salt

25

A 0.1 mmol sample of the free acid of a compound of formula (I) is dissolved in 10 mL ethyl acetate. The resulting solution is saturated with gaseous ammonia and the ammonium salt precipitates from solution.

EXAMPLE 12

Preparation of a Potassium Salt

30

A solution of 0.1 mmol of the free acid of a compound of formula (I) in 10 mL methanol is treated with an aqueous or methanolic solution containing 0.3 mmol of potassium hydroxide. Evaporation of the solvent affords the tri-potassium salt. Addition of between 0.1 and 0.3 mmol of potassium hydroxide yields analogously mixtures of the

- 41 -

mono-potassium, di-potassium and tri-potassium salts whose composition depends upon the exact amount of potassium hydroxide added.

In a similar fashion, the sodium and lithium salts can be formed.

EXAMPLE 13

Preparation of a Calcium Salt

A solution of 0.1 mmol of the free acid of a compound of formula (I) in 20 mL 6:4 methanol:water is treated with an aqueous solution of 0.1 mmol of calcium hydroxide. The solvents are evaporated to give the corresponding calcium salt.

EXAMPLE 14

Preparation of an Ethylenediamine Salt

A solution of 0.1 mmol of the free acid of a compound of formula (I) in 10 mL of methanol is treated with 0.1 mmol of ethylenediamine. Evaporation of the solvent affords the ethylenediamine salt.

The procedure can also be applied to the preparation of the N,N"-dibenzylethylenediamine salt.

EXAMPLE 15

Preparation of a Tris(hydroxymethyl)aminomethane Salt

To a solution of 0.1 mmol of the free acid of a compound of formula (I) in 10 mL of methanol is added from 0.1 to 0.3 mmol of tris(hydroxymethyl)aminomethane dissolved in 10 mL methanol. Evaporation of the solvent gives a corresponding salt form, the exact composition of which is determined by the molar ratio of amine added. Similarly prepared are the salts of L-ornithine, L-lysine, and N-methylglutamine.

- 42 -

EXAMPLE 16

Preparation of an L-arginine Salt

5 A solution of 0.1 mmol of the free acid of a compound of formula (I) in 20 mL of 6:4 methanol: water is treated with an aqueous solution of 0.1 to 0.3 mmol of L-arginine. Evaporation of the solvent affords the title salt, the exact composition of which is determined by the molar ratio of amino acid to the free acid of formula (I) used.

10 Similarly prepared are the salts of L-ornithine, L-lysine and N-methylglutamine.

EXAMPLE 17

Preparation of a benzyl ester

15 A solution of 5 mg of the free acid of a compound of structural formula (I), in 0.5 mL of tetrahydrofuran (THF) is treated at room temperature with 3 equivalents of N,N'-diisopropyl-O-benzyl isourea for 18 hours. The reaction mixture is then chilled to -15°C, filtered to remove the urea. The filtrate is concentrated under reduced pressure to yield Compound B.

20 This method is also suitable for the preparation of other ester derivatives such as: 1) methyl and the other lower alkyls, and 2) substituted benzyl esters, using the appropriately substituted isourea. By varying the number of equivalents of the substituted isourea used, the mono-, di- and tri-substituted esters may be selectively prepared.

Mass Spectral Data

25 Mass spectra were recorded on Finnigan-MAT model MAT212 (Electron Impact, EI, 90 eV), TSQ70B (Fast Atom Bombardment, FAB, EI 70eV), and Jeol SX102 (EI, 90eV, FAB) mass spectrometers. Electron impact exact mass measurements were performed at high resolution (HR-EI) using perfluorokerosene (PFK) as internal standard. Fast Atom Bombardment (FAB) exact mass

- 43 -

measurements were performed at high resolution (HR-FAB) using polyethylene glycol (PEG)600 as internal standard.

¹³C NMR Data

5 ¹³C NMR spectra were recorded in CD₃OD at 75 MHz on a Varian XL-300 spectrometer or at 100 MHz on a Varian Unity 400 spectrometer. Chemical shifts are given in ppm relative to the solvent peak at 49.0 ppm (CD₃OD) as internal standard.

¹H NMR Spectra

10 ¹H NMR spectra were recorded at 300 MHz on a Varian XL-300 spectrometer and/or at 400 MHz or 500 MHz on a Varian Unity spectrometer. Chemical shifts are shown in ppm relative to the solvent peaks at 3.30 ppm (CD₃OD) as internal standards.

Physical Properties of the compounds of Structure I:

15 Compound E - The compound of structure (I) wherein R₁ is p-hydroxybenzyl, R₂ is H, R₃ is H, R₄ is H, R₅ is OZ₃, R₆ is methyl, R₇ is -N(R₄)CH(R₁)C(=O)OZ₁, and Z₁, Z₂ and Z₃ are each H.

20 Mass Spectral Data: This compound has the molecular weight of 577 by FAB-MS (observed [M-H]⁻ at m/z 576 and [M+H]⁺ at m/z 578). HR-FAB indicates a molecular formula of C₃₁H₄₇O₉N (found 578.3304, calculated 578.3329, for C₃₁H₄₈O₉N, [M+H]⁺).

25 ¹H nmr (400 MHz, 10 mg in 0.7 mL CD₃OD): δ 0.89 (t, 7.2, 3H), 1.27 (m, 24H), 1.97 (m, 2H), 2.61 (d, 16.4, 1H), 2.89 (dd, 8.8, 14.0 1H), 2.90 (d, 15.6, 1H), 3.10 (dd, 5.2, 14.4, 1H), 3.20 (d, 8.0, 1H), 4.60 (dd, 4.8, 8.8, 1H), 5.54 (m, 2H), 6.67 (d, 8.8, 2H), 7.01 (d, 8.8, 2H).

30 ¹³C nmr (100 MHz, 10 mg in 0.7 mL CD₃OD): δ 14.45, 23.74, 30.16, 30.24, 30.48, 30.62, 30.77(6), 33.08, 33.61, 37.51, 43.07, 55.07, 57.83, 77.93, 116.20(2), 124.35, 128.67, 131.36(2), 137.73, 157.36, 173.48, 173.80, 174.43, 175.67.

- 44 -

Compound F - The compound of structure (I) wherein R₁ is 3-indolyl, R₂ is H, R₃ is OH, R₄ is H, R₅ is OZ₃, R₆ is methyl, R₇ is -N(R₄)CH(R₁)C(=O)OZ₁, and Z₁, Z₂ and Z₃ are each H.

Mass Spectra Data: This compound has the molecular weight of 616 by FAB-MS (observed [M-H]⁻ at m/z 615 and [M+H]⁺ at m/z 617). HR-FAB indicates a molecular formula of C₃₃H₄₈O₉N₂ (found 617.3362, calculated 617.3438, for C₃₃H₄₉O₉N₂, [M+H]⁺).

¹H nmr (500 MHz, 2 mg in 0.7 mL CD₃OD): δ 0.90 (t, 6.0, 3H), 1.30 (m, 12H), 1.41 (m, 2H), 1.53 (m, 2H), 1.93 (m, 2H), 2.57 (d, 16.2, 1H), 2.87 (d, 16.1, 1H), 3.19 (d, 8.2, 1H), 3.21 (dd, 7.5, 15.0, 1H), 3.33 (dd, 5.5, 15.0, 1H), 3.48 (m, 1H), 4.74 (dd, 4.9, 7.7, 1H), 5.53 (m, 2H), 6.99 (ddd, 1.1, 7.0, 8.1, 1H), 7.06 (ddd, 1.1, 6.8, 8.0, 1H), 7.10 (s, 1H), 7.30 (dt, 0.8, 8.1, 1H), 7.55 (dt, 1.0, 8.0).

UV (MeOH): 221 nm, 281 nm, 290 nm.

Compound G - The compound of structure (I) wherein R₁ is p-hydroxybenzyl, R₂ and R₃ together are oxo, R₄ is H, R₅ is OZ₃, R₆ is n-propyl, R₇ is -N(R₄)CH(R₁)C(=O)OZ₁, and Z₁, Z₂ and Z₃ are each H.

Mass Spectral Data: This compound has the molecular weight of 619 by FAB-MS (observed [M-H]⁻ at m/z 618 and [M+H]⁺ at m/z 620). HR-EI on the trimethyl ester indicates a molecular formula of C₃₃H₄₉O₁₀N (found = 625.3642, calculated = 625.3614 for C₃₆H₅₁O₈N, C₃₃H₄₉O₁₀N + 3xCH₂ - H₂O - H₂O).

¹H nmr (500 MHz, 1.0 mg in 0.7 mL CD₃OD): δ 0.89 (t, 6.5, 3H), 1.28 (m, 18H), 1.52 (m, 4H), 1.98 (m, 2H), 2.42 (t, 7.5, 2H), 2.43 (t, 7.5, 2H), 2.59 (d, 16.0, 1H), 2.86 (d, 16.0, 1H), 2.90 (dd, 8.5, 14.0, 1H), 3.09 (dd, 5.0, 14.0, 1H), 3.20 (d, 8.5, 1H), 4.59 (dd, 5.0, 8.0, 1H), 5.54 (m, 2H), 6.66 (d, 9.0, 2H), 7.03 (d, 9.0, 2H).

Compound H - The compound of structure (I) wherein R₁ is p-hydroxybenzyl, R₂ and R₃ together are oxo, R₄ and R₅ together are a single bond, R₆ is methyl, R₇ is -N(R₄)CH(R₁)C(=O)OZ₁, and Z₁, Z₂ and Z₃ are each hydrogen.

- 45 -

Mass Spectral Data: This compound has the molecular weight of 573 by FAB-MS (observed $[M-H]^-$ at m/z 572 and $[M+H]^+$ at m/z 574). HR-FAB indicates a molecular formula of $C_{31}H_{43}O_9N$ (found = 574.3019, calculated = 574.3015 for $C_{31}H_{44}O_9N$, $[M+H]^+$).

5 1H nmr (300 MHz, 13.6 mg in 0.7 mL CD_3OD): δ 0.90 (t, 6.5, 3H), 1.30 (m, 14H), 1.54 (m, 4H), 1.99 (m, 2H), 2.45 (t, 7.2, 4H), 2.57 (AB quartet, 2H), 3.18 (dd, 10.3, 14.3, 1H), 3.27 (d, 8.6, 1H), 3.37 (dd, 5.7, 14.3 1H), ~ 4.9 (obscured under HDO peak, 1H), 5.15 (ddt, 1.3, 8.8, 15.5, 1H), 5.70 (dt, 7.0, 15.3, 1H), 6.66 (d, 8.5, 2H), 6.98 (d, 8.4, 2H).

10 ^{13}C nmr (75 MHz, 13.6 mg in 0.7 mL CD_3OD): δ 14.22, 23.68, 24.81, 24.92, 29.71, 29.87, 30.02, 30.25, 30.29, 32.89, 33.56, 34.28, 38.86, 43.44, 43.50, 55.24, 57.13, 76.87, 116.23 (2), 121.43, 129.24, 131.26 (2), 140.62, 157.30, 171.60, 173.16, 176.93, 178.44, 15 214.52.

The characterization of the Compounds isolated in Examples 4 through 10 are detailed in Table 2 which follows:

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25

30

TABLE 2

Fraction	Mwt	Formula	Found [M+H] ⁺	Calculated	For	RRT/Solvent System	Structure
20	607	C ₃₁ H ₄₅ NO ₁₁	608.3105	608.3070	C ₃₁ H ₄₆ NO ₁₁	-	-
	589	C ₃₁ H ₄₃ NO ₁₀	590.3049	590.2964	C ₃₁ H ₄₄ NO ₁₀	-	-
	609	C ₃₁ H ₄₇ NO ₁₁	610.3225	610.3227	C ₃₁ H ₄₈ NO ₁₁	-	-
	573	C ₃₁ H ₄₃ NO ₉	574.3038	574.3015	C ₃₁ H ₄₄ NO ₉	-	-
22	589	C ₃₁ H ₄₃ NO ₁₀	590.3018	590.2964	C ₃₁ H ₄₄ NO ₁₀	-	-
	609	C ₃₁ H ₄₇ NO ₁₁	610.3259	610.3227	C ₃₁ H ₄₈ NO ₁₁	-	-
	573	C ₃₁ H ₄₃ NO ₉	574.3049	574.3015	C ₃₁ H ₄₄ NO ₉	-	-
							Cmpd. P
24	589	C ₃₁ H ₄₃ NO ₁₀	590.2986	590.2964	C ₃₁ H ₄₄ NO ₁₀	-	-
	609	C ₃₁ H ₄₇ NO ₁₁	610.3176	610.3227	C ₃₁ H ₄₈ NO ₁₁	-	-
	607	C ₃₁ H ₄₅ NO ₁₁	608.3024	608.3070	C ₃₁ H ₄₆ NO ₁₁	-	-
	605	C ₃₁ H ₄₃ NO ₁₁	606.2904	606.2914	C ₃₁ H ₄₄ NO ₁₁	-	-
	573	C ₃₁ H ₄₃ NO ₉	574.3049	574.3015	C ₃₁ H ₄₄ NO ₉	-	-
26	609	C ₃₁ H ₄₇ NO ₁₁	610.3176	610.3227	C ₃₁ H ₄₈ NO ₁₁	-	-
	607	C ₃₁ H ₄₅ NO ₁₁	608.3058	608.3070	C ₃₁ H ₄₆ NO ₁₁	-	-
	605	C ₃₁ H ₄₃ NO ₁₁	606.2904	606.2914	C ₃₁ H ₄₄ NO ₁₁	-	-
	589	C ₃₁ H ₄₃ NO ₁₀	590.2988	590.2964	C ₃₁ H ₄₄ NO ₁₀	-	-
	573	C ₃₁ H ₄₃ NO ₉	574.3011	574.3015	C ₃₁ H ₄₄ NO ₉	-	-

SUBSTITUTE SHEET (RULE 26)

TABLE 2 (CONT'D)

Fraction	Mwt	Formula	Found [M+H] ⁺	Calculated	For	RRT/Solvent System	Structure
32	607	C ₃₁ H ₄₅ NO ₁₁	608.3044	608.3070	C ₃₁ H ₄₆ NO ₁₁	-	-
	605	C ₃₁ H ₄₃ NO ₁₁	606.2858	606.2914	C ₃₁ H ₄₄ NO ₁₁	-	-
	589	C ₃₁ H ₄₃ NO ₁₀	590.2988	590.2987	C ₃₁ H ₄₄ NO ₁₀	-	-
	567	C ₂₉ H ₄₅ NO ₁₀	568.3093	568.3121	C ₂₉ H ₄₆ NO ₁₀	-	-
	565	C ₂₉ H ₄₃ NO ₁₀	566.2981	566.2964	C ₂₉ H ₄₄ NO ₁₀	-	-
44	577	C ₃₀ H ₄₃ NO ₁₀	578.2999	578.2965	C ₃₀ H ₄₄ NO ₁₀	-	Cmpd. O
	593	C ₃₁ H ₄₇ NO ₁₀	594.3224	594.3278	C ₃₁ H ₄₈ NO ₁₀	-	-
48	593	C ₃₁ H ₄₇ NO ₁₀	594.3279	594.3278	C ₃₁ H ₄₈ NO ₁₀	-	Cmpd. N
50-64	591					1.0 / B,C	Cmpd. A
65-73	630	C ₃₃ H ₄₆ N ₂ O ₁₀	631.3188	631.3230	C ₃₃ H ₄₇ N ₂ O ₁₀	-	-
74-77	616	C ₃₃ H ₄₈ N ₂ O ₉	617.3362	617.3438	C ₃₃ H ₄₉ N ₂ O ₉	-	Cmpd. F
	559	C ₃₁ H ₄₅ NO ₈	560.3193	560.3223	C ₃₁ H ₄₆ NO ₈	-	Cmpd. Q
78-79	577	C ₃₁ H ₄₇ NO ₉	578.3313	578.3329	C ₃₁ H ₄₈ NO ₉	-	Cmpd. S
	561	C ₃₀ H ₄₃ NO ₉	562.3058	562.3016	C ₃₀ H ₄₄ NO ₉	-	Cmpd. R
80-83	614	C ₃₃ H ₄₆ N ₂ O ₉	615.3214	615.3281	C ₃₃ H ₄₇ N ₂ O ₉	-	Cmpd. D
86-90	575	C ₃₁ H ₄₅ NO ₉	576.3160	576.3172	C ₃₁ H ₄₆ NO ₉	-	Cmpd. C

SUBSTITUTE SHEET (RULE 26)

TABLE 2 (CONT'D)

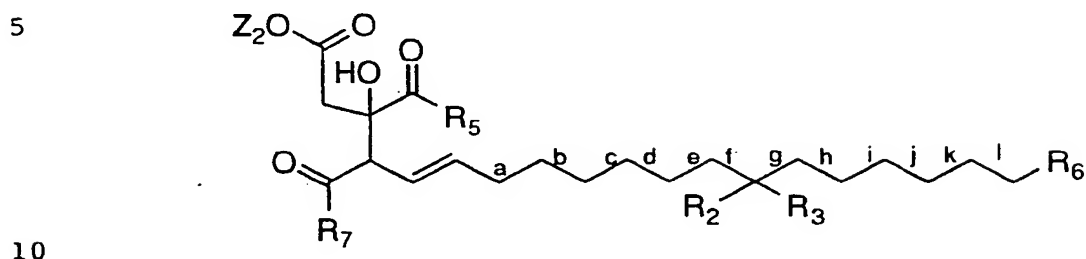
Fraction	Mwt	Formula	Found [M+H] ⁺	Calculated	For	RRT/Solvent System	Structure
108-110	577	C ₃₁ H ₄₇ NO ₉	578.3304	578.3329	C ₃₁ H ₄₈ NO ₉	-	Cmpd. E
111-119	603	C ₃₃ H ₄₉ NO ₉	604.3450	604.3485	C ₃₃ H ₅₀ NO ₉	-	Cmpd. M
	579	C ₂₇ H ₄₉ NO ₁₂	580.3333	580.3332	C ₂₇ H ₅₀ NO ₁₂	-	
	577	C ₂₇ H ₄₇ NO ₁₂	578.3213	578.3176	C ₂₇ H ₄₈ NO ₁₂	-	
136-143	561	C ₃₁ H ₄₇ NO ₈	562.3354	562.3379	C ₃₁ H ₄₈ NO ₈	-	Cmpd. L
	428	C ₂₃ H ₄₀ O ₇	429.2863	429.2852	C ₂₃ H ₄₁ O ₇	-	Cmpd. T

SUBSTITUTE SHEET (RULE 26)

- 49 -

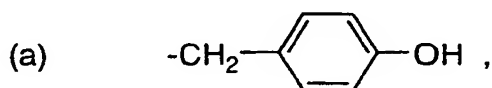
WHAT IS CLAIMED IS:

1. A compound of structural formula (I)

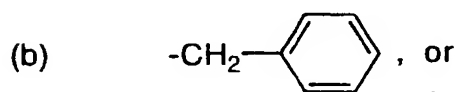


wherein
R₁ is

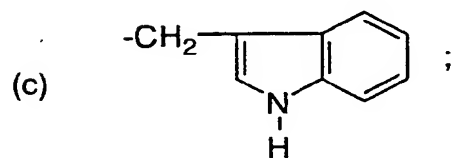
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R₂ and R₃ are each H, or R₂ is H and R₃ is OH, or R₂ and R₃ together are oxo;

R₄ is H and R₅ is OZ₃, or R₄ and R₅ are a single bond forming a 2,5-dioxopyrrolidine;

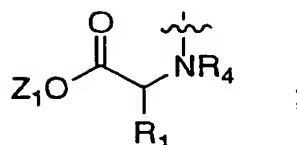
- 50 -

R₆ is

- (a) hydrogen, or
- (b) C₁₋₃ alkyl;

R₇ is:

- (a) -OZ₁, or
- (b)



each of a, b, c, d, e, f, g, h, i, j, k, and l are a single bond or one of a, b, c, d, e, f, g, h, i, j, k, and l is a double bond, provided that when f or g is a double bond, R₂ is absent and R₃ is H; and

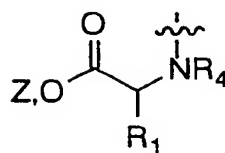
Z₁, Z₂ and Z₃ are each independently

- (a) H,
- (b) C₁₋₅ alkyl, or
- (c) C₁₋₅ alkyl substituted with
 - (i) phenyl, or
 - (ii) phenyl substituted with methyl, methoxy, Cl, Br, I, F or hydroxy;

or a pharmaceutically acceptable salt of a compound of formula (I) provided that when

R₂ and R₃ are together oxo,
 R₄ is H,
 R₅ is OZ₃,
 R₆ is methyl, and
 R₇ is

- 51 -



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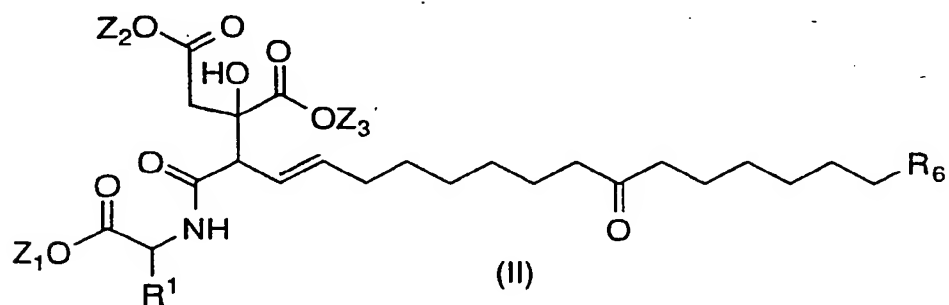
one of a, b, c, d, e, f, g, h, i, j, k, and l is a double bond.

2. The compound of Claim 1 in which R₇ is
 -N(R₄)CH(R₁)C(=O)OZ₁, and Z₁, Z₂ and Z₃ are each hydrogen or a
 10 pharmaceutically acceptable mono, di or tri salt thereof.

3. The compound of Claim 1 of structural formula (II):

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wherein R₁, R₆, Z₁, Z₂ and Z₃ are:

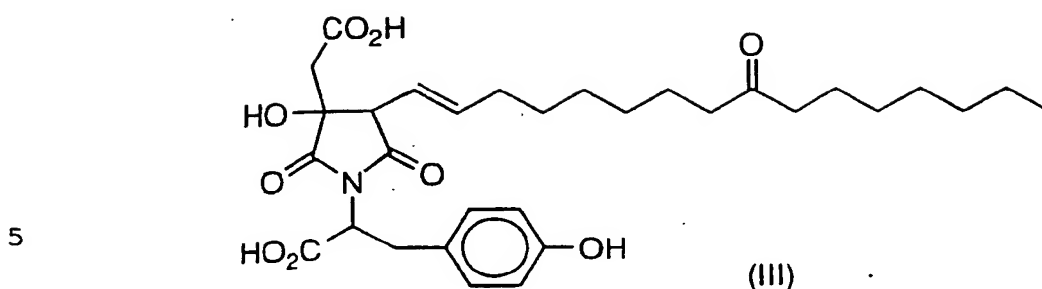
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	R ₁	R ₆	Z ₁	Z ₂	Z ₃
(a)	p-hydroxybenzyl	n-propyl	H	H	H
(b)	benzyl	hydrogen	H	H	H
(c)	benzyl	propyl	H	H	H
(d)	p-hydroxybenzyl	hydrogen	H	H	H

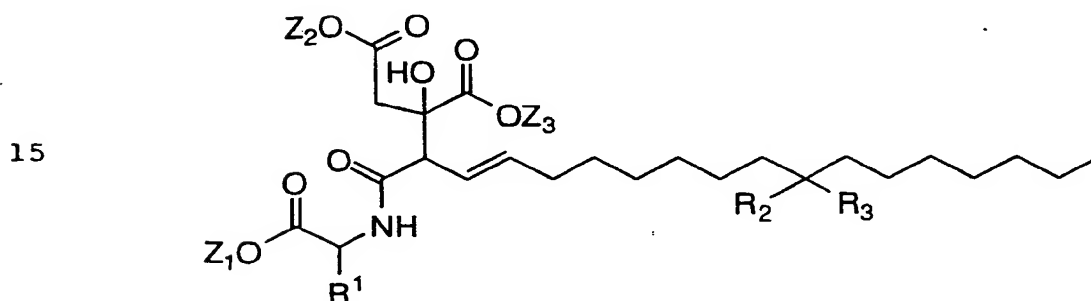
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4. The compound of Claim 1 of structural formula
 (III):

- 52 -



10 5. The compound of Claim 1 of structural formula (IV):



wherein R₁, R₂, R₃, Z₁, Z₂ and Z₃ are:

	R ₁	R ₆	R ₃	Z ₁	Z ₂	Z ₃
(a)	3-indolyl	H	OH	H	H	H
(b)	p-hydroxybenzyl	H	H	H	H	H
25 (c)	benzyl	H	OH	H	H	H
(d)	benzyl	H	H	H	H	H

6. The compound according to Claim 1 wherein Z₁ is OH, Z₂ is OH, Z₃ is OH, R₄ is H, R₅ is OH, R₇ is
 30 -N(R₄)CH(R₁)C(=O)OZ₁ and R₁, R₂, R₃, and R₆ are as below:

- 53 -

	R ₁	R ₂	R ₃	R ₆
	(a) benzyl	-H	-H	-CH ₃
5	(b) benzyl	oxo with R ₃	oxo with R ₂	-CH ₂ CH ₂ CH ₃
	(c) p-hydroxybenzyl	-H	-OH	-CH ₃
	(d) p-hydroxybenzyl	oxo with R ₃	oxo with R ₂	-H
10	(e) benzyl	oxo with R ₃	oxo with R ₂	-H
	(f) benzyl	-H	-OH	-CH ₃ .

15 7. The compound of Claim 1 wherein R₇ is -N(R₄)CH(R₁)C(=O)OZ₁, R₄ is hydrogen, R₅ is OZ₃, Z₁, Z₂ and Z₃ are each hydrogen, and one of a, b, c, d, e, f, g, h, i, j, k, and l is a double bond provided that when f or g is a double bond, and R₁, R₂, R₃, and R₆ are as shown in the table below:

20

	R ₁	R ₂	R ₃	R ₆
	(a) benzyl	H	H	methyl
25	(b) p-hydroxybenzyl	oxo with R ₃	oxo with R ₂	methyl.

8. The compound of Claim 1 wherein R₇ is -OZ₁.

30 9. A process of making a compound of Claim 1 wherein Z₁, Z₂, and Z₃ are each H, comprising cultivating Trichoderma viride (ATCC 74084), or a mutant thereof, under conditions suitable for the formation of the compound and recovering the compound.

- 54 -

10. A biologically pure culture of Trichoderma viride (ATCC 74084) capable of forming the compound of Claim 1 in recoverable amounts.

5 11. A pharmaceutical composition comprising a nontoxic therapeutically effective amount of a compound of Claim 1 and a pharmaceutically acceptable carrier.

10 12. A method of treating hypercholesterolemia comprising the administration to a subject in need of such treatment a nontoxic therapeutically effective amount of a compound of Claim 1.

15 13. A method of inhibiting squalene synthase comprising the administration of a subject in need of such treatment a nontoxic therapeutically effective amount of a compound of Claim 1.

14. A method for inhibiting fungal growth comprising applying to the area where growth is to be controlled an antifungally effective amount of a compound of Claim 1.

20 15. A method for inhibiting fungal growth in a living organism in need of such treatment comprising the oral, systemic, topical or parenteral administration to the living organism of an antifungally effective amount of the compound as defined in Claim 1 or
25 a pharmaceutically acceptable salt thereof.

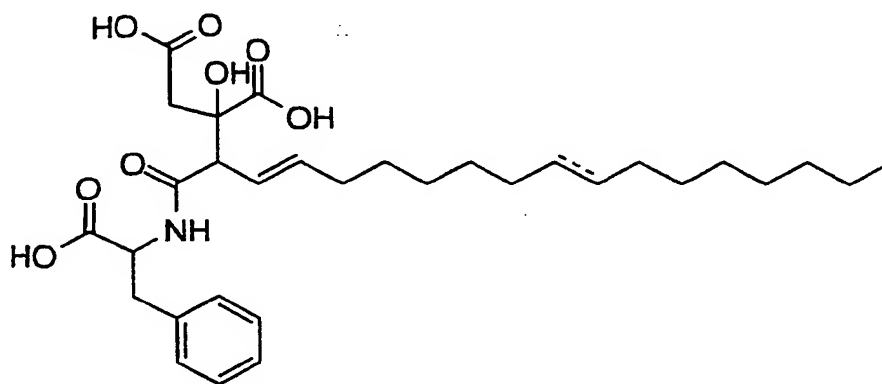
16. The method of Claim 15 wherein the living organism is a vertebrate.

30 17. The method of Claim 15 wherein the living organism is a mammal.

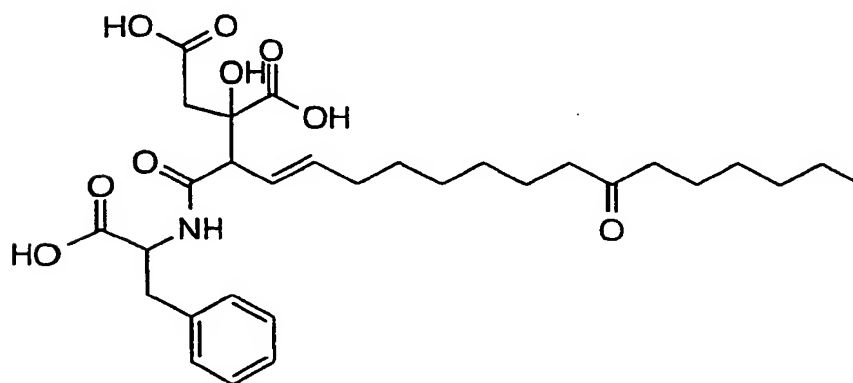
18. The method of Claim 15 wherein the living organism is a bird.

20. The compound according to Claim 1 selected from:

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(b)



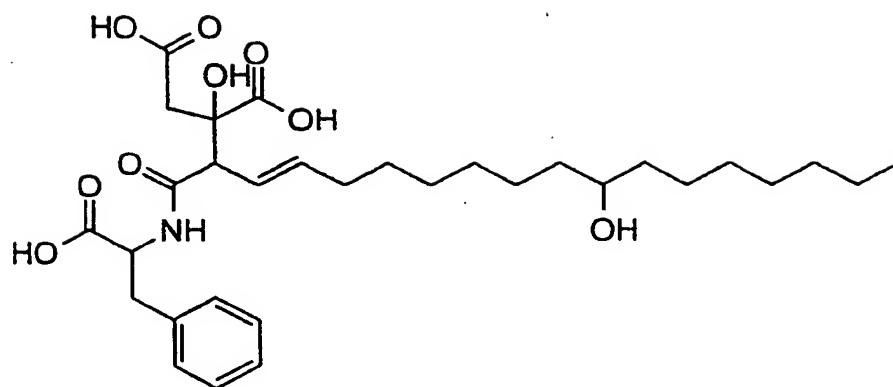
BNSDOCID: <WO_____9418157A1_1_>

- 56 -

(c)

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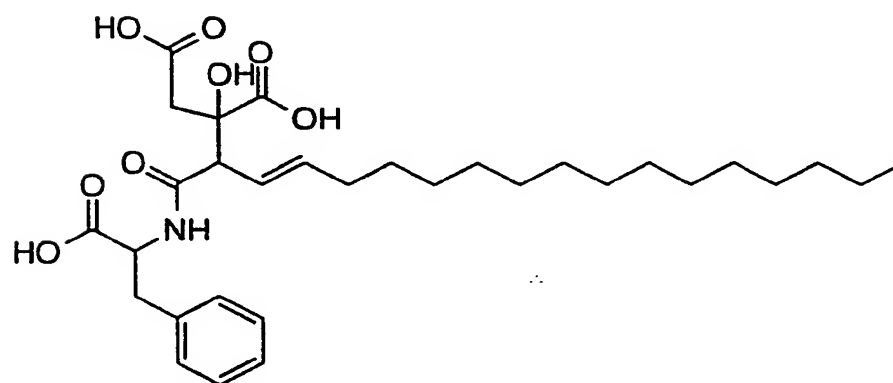
(d)

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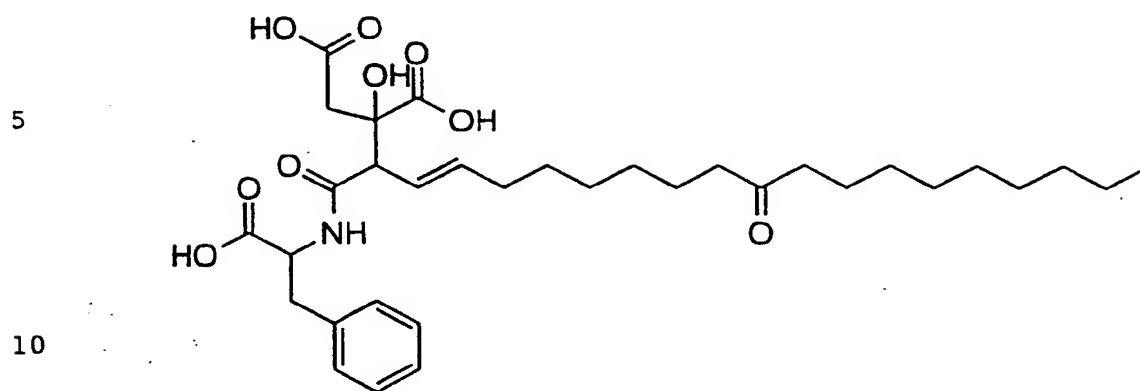
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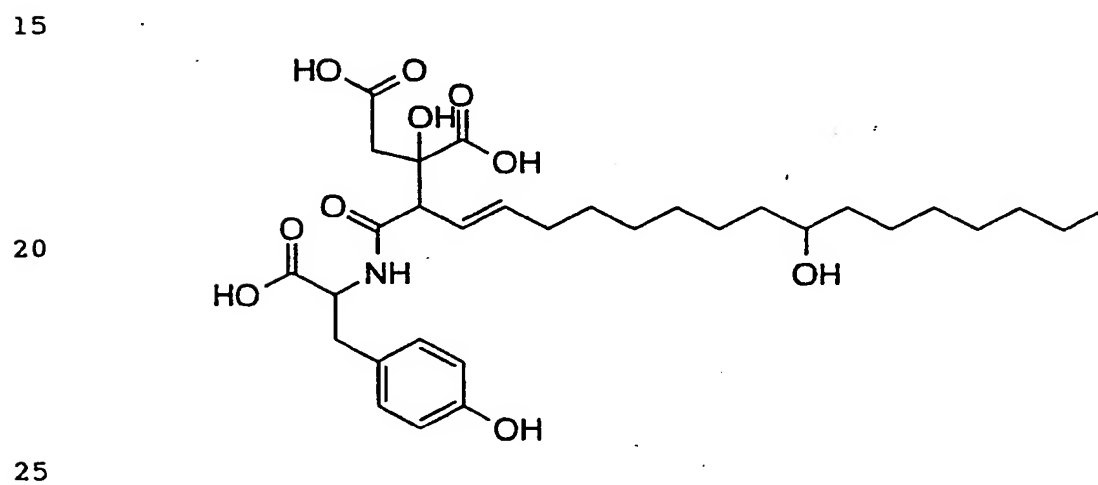


- 57 -

(e)



(f)



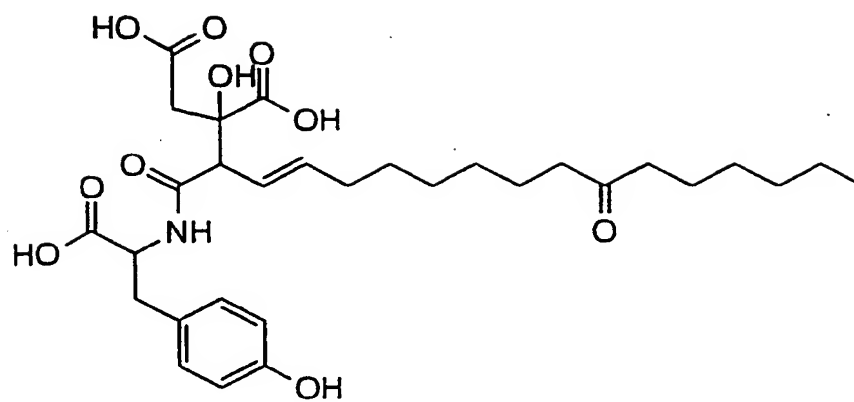
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- 58 -

(g)

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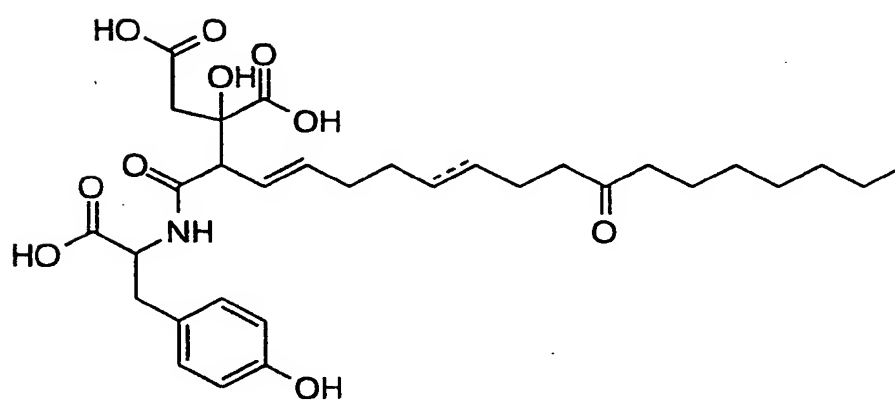


(h)

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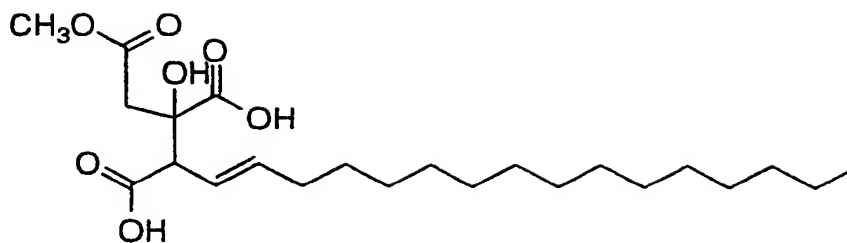
, and

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- 59 -

(i)

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wherein: the dashed line indicates the presence of a double bond along the alkenyl chain.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/00792

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07C 233/00
US CL :554/36,61,63,213,218,227,228,229; 548/469,534; 435/41,254.6,536,945
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 554/36,61,63,213,218,227,228,229; 548/469,534; 435/41,254.6,536,945

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, CAS Online

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 4,603,142 (Burger et al.) 29 July 1986, entire document.	1-12, 20
A	US, A, 5,182,298 (Helms et al.) 26 January 1993, entire document.	1-12, 20
A,P	US, A, 5,244,795 (Dombroski et al.) 14 September 1993, entire document.	1-12, 20
A,P	US, A, 5,245,050 (Endo et al.) 14 September 1993, entire document.	1-12, 20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

18 MAY 1994

Date of mailing of the international search report

MAY 26 1994

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

DEBORAH D. CARR

Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/00792

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

I. Claims 1-12, 20, drawn to the compound, method of making, and method of using.

II. Claim 13, drawn towards a second method of using the compound.

III. Claim 14, drawn towards a third method of using the compound.

IV. Claims 15-19, drawn towards a fourth method of using the compound.

The application contains one or more of the recognized categories that define unity of invention. As shown in the groupings above, the application contains claims that read on a compound, process of making and multiple methods of using, 37 CFR 1.475(d).

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/00792

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims. It is covered by claims Nos.:
1-12 & 20

Remark on Protest

☐
☐

The additional search fees were accompanied by the applicant's protest.
No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)*